111. (new) The immunoconjugate of claim 110, wherein the ribosome binding toxin is ricin A.

112. (new) The immunoconjugate of claim 110, wherein the therapeutic agent is an exotoxin.

113. (new) The immunoconjugate of claim 112, wherein the exotoxin is Pseudomonas exotoxin A.

114. (new) The immunoconjugate of claim 112, wherein the exotoxin is truncated to remove the cell-binding domain.

115. (new) The immunoconjugate of claim 112, wherein the amino terminus of the exotoxin has been modified to include a lysine amino acid residue.

116. (new) The immunoconjugate of claim 109 which is purified.

117. (new) A pharmaceutical composition, comprising a pharmaceutically effective amount of the immunoconjugate of claim 101, 102, 105, 106, or 107 and a pharmaceutically acceptable carrier.

118. (new) The pharmaceutical composition of claim 117 wherein the immunoconjugate is purified.

REMARKS

The Attorney Docket Number for the above-identified application has been changed to "9632-033." It is respectfully requested that the computer records at the United States Patent and Trademark Office be amended and that the current Attorney Docket Number be used on all papers for this file.

Claims 59-65, 71, 84 and 93 were pending in the instant application. Claims 61 and 63 have been amended and new claims 94-118 have been added to more clearly claim

that which Applicants regard as the invention. The amendment and new claims find support throughout the specification and therefore do not introduce new matter. Support for the amendment to claim 61 may be found at page 44, line 24 to page 45, line 5. Support for the new claims may be found in the specification, for example, as follows:

Claim 94 finds support at page 35, lines 4 to 11, page 40, lines 36 to 41, and page 54, lines 18 to 35. Claim 95 finds support at page 41, lines 14 to 17, and page 54, lines 20 to 35. Claim 96 finds support at page 55, lines 3 to 19, and page 61, lines 9 to 19. Claim 97 finds support at page 55, lines 1 to 2, and page 61, lines 9 to 19. Claim 98 finds support at page 57, lines 10 to 20. Claim 99 finds support at page 54, lines 15 to 17. Claim 100 finds support at page 73, line 24 to 30. Claim 101 finds support at page 26, lines 8 to 11, page 37, line 30 to page 38, line 18. Claim 102 finds support at page 26, line 8 to 11, and page 37, line 30 to page 38, line 18. Claim 103 finds support at page 54, lines 20 to 35. Claim 104 finds support at page 55, line 1 to page 56, line 4. Claim 105 finds support at page 55, lines 1 to 2. Claim 106 finds support at page 57, lines 10 to 20. Claim 107 finds support at page 54, lines 15 to 17. Claim 108 finds support at page 73, lines 24 to 30. Claim 109 finds support at page 27, lines 1 to 19; page 44, line 14 to page 45, line 28; and page 53, line 9 to page 54, line 19. Claims 110-113 find support at page 44, lines 25 to 31. Claim 114 and 115 find support at page 44, line 24 to page 45, line 28. Claim 116 finds support at page 37, line 30 to page 38, line 18. Claim 117 finds support at page 30, lines 17-35. Claim 118 finds support at page 30, lines 17-35 and page 73, lines 24-30.

The Rejections Under 35 U.S.C. § 103 (a) Should be Withdrawn

Claims 59-65, 71, 84 and 93 are rejected under 35 U.S.C. § 103(a), allegedly as being obvious over Abe *et al.*, 1986, Cancer Res. 46:2639-44 ("Abe"); Kim *et al.*, 1986, Cancer Res. 46:5985-5992 ("Kim"); and Hellström *et al.*, 1986, Cancer Res. 46:3917-3923 ("Hellström"), in view of the teachings of Oldham *et al.*, 1983, J. Biol. Resp. Modif. 2:1-37 ("Oldham") and Schlom, in "Molecular Foundations of Oncology," Broder, Ed., Williams and Wilkins, 1991 pp. 95-134 ("Schlom"), to make the claimed invention. The Examiner alleges that the teaching of the prior art, when combined, provide the suggestion and the motivation to grant one of ordinary skill in the art the reasonable expectation of success in arriving at the claimed invention.

In response, Applicants respectfully disagree. Applicants assert that the Examiner has not established a *prima facie* case of obviousness, wherein which the Examiner must demonstrate: (1) that the combined references teach or suggest all claim limitations; (2) these references provide teaching, suggestion or motivation to combine or modify the teaching of the prior art to produce the claimed invention; and (3) the combined teaching of the prior art references indicates a reasonable expectation of success in arriving at the claimed invention. *See, e.g., In re Mayne*, 104 F.3d 1339, 1341-42, 41 U.S.P.Q.2d 1451 (Fed. Cir. 1997). Additionally, when a rejection for alleged *prima facie* obviousness relies upon a combination of prior art references, the burden is upon the Examiner to establish that there exists a teaching, motivation, or suggestion to combine the prior art references, and that this art suggests the desirability of doing so. *In re Rouffet*, 149 F.3d 1350, 1355, 47 U.S.P.Q.2d 1453 (Fed. Cir. 1998); *In re Fritch*, 922 F.2d 1260, 23 U.S.P.Q.2d 1790 (Fed. Cir. 1992).

The prior art references cited by the Examiner fail to describe or suggest an immunoconjugate that comprises a Lewis Y specific antibody that is internalized by a carcinoma cell with which it reacts. Abe, Kim, and Hellstrom make no mention of immunoconjugates and do not discuss internalization within cells; Oldham describes the use of monoclonal antibodies as a component of immunoconjugates, but does not contemplate internalization of such immunoconjugates within cells. Examiner alleges that a single cited reference, Schlom, provides the rationale and the motivation that the development of monoclonal antibody conjugates that would be able to internalize within a cell would be desirable.

Applicants respectfully point out that, even assuming *arguendo* that Schlom teaches that such a goal is desirable, Schlom does not provide sufficient teaching or motivation so that one of ordinary skill in the art would reasonably expect to achieve success with this goal, independent of its desirability. Rather, Schlom, in observing "that some solid tumor membrane antigens are stable cell surface components suggests that a subset of mAb drug conjugates will be ineffective against these target antigens" (p. 107) and that the "necessity of the mAb conjugate to internalize for cytotoxic activity" provides a "theoretical limitation to the potential usefulness of mAb drug or toxin conjugates as effective oncolytic agents" (p. 107) instead demonstrates that one of ordinary skill would <u>not</u> reasonably expect to successfully develop an immunoconjugate capable of internalizing within a carcinoma cell.

Schlom, along with the other references cited by the Examiner, do not suggest with a reasonable expectation of success that an antibody (or conjugate thereof) to a Lewis Y antigen would internalize within the carcinoma cells displaying the Lewis Y antigen. Applicants note that the internalization of antibodies within cells is highly unpredictable in nature and thus the supposition that one of ordinary skill in the art would reasonably expect that immunoconjugates specifically targeted for Lewis Y antigens would be internalized by the cell, without benefit of the teachings of Applicant's disclosure, is unfounded.

Applicants point out that the desirability of a research goal, and the notion that efforts leading to a desirable goal as "obvious to try" have long been considered improper grounds for an obviousness rejection. *See, e.g., In re O'Farrell*, 853 F.2d 894, 903 (Fed. Cir. 1988); *see also* MPEP § 2145 (X)(b). Examiner's arguments that "one of ordinary skill would have known at the time of the claimed invention how to screen for ligands that internalized into cells" and such efforts would be motivated to do so as "[i]nternalizing antibodies are desirable in those instances where there would have been a necessity for introducing the cytotoxic, diagnostic or therapeutic agent to the interior of the cell" do not generate a reasonable expectation of success and rather constitute merely an argument that the present invention was "obvious to try." In fact, this argument embodies one type of error perpetrated by improper "obvious to try" rejections that the Federal Circuit expressed specific concerns to avoid in the decision of *In re O'Farrell*. As the Federal Circuit explained:

> The admonition that "obvious to try" is not the standard under § 103 has been directed mainly at two kinds of error. In some cases, what would have been "obvious to try" would have been to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful. *See id.* at 903 (emphasis added).

Applicants assert that direction as to which choices are likely to be successful is especially critical in art of a highly unpredictable nature, such as the internalization of antibodies within target cells. *See In re Rinehart*, 531 F.2d 1048, 189 U.S.P.Q. 143 (CCPA 1976) (evidence showing the lack of reasonable expectation of success in an art of limited predictability may support a conclusion of nonobviousness). It is known in the art that not all antibodies are internalized. For example, researchers have identified anti-transferrin receptor antibodies that fail to demonstrate internalization upon binding to the target cell. *See* Exhibit

C (Lesley *et al.*, 1989, Exptl. Cell Research 182:215-233). Similar findings have also been demonstrated with antibodies to the Human CSF-1 receptor (which antibodies are presumed not to internalize since their cognate receptor is not internalized upon antibody binding). *See* Exhibit D (Sherr *et al.*, 1989, Blood 73(7):1786-1793). Researchers conducting studies on the internalization of antibodies to malignant B-cells have noted the substantial variance in internalization results. *See* Exhibit E (Press *et al.*, 1989, Cancer Research 49(17):4906-4912). Similarly, Berinstein *et al.* have shown that antibody binding to a cell is insufficient to guarantee internalization. *See* Exhibit F (Berinstein *et al.*, 1987, Cancer Research 47(22):5954-5959). Furthermore, Berinstein *et al.* note that the rate of endocytosis of the antigen-immunotoxin complex is a major parameter influencing effective growth inhibition by immunotoxins, and may limit the use of their studied immunotoxin for cytotoxic purposes to certain targeting antibodies. *See id.*, at 5958-59.

As internalization of antibodies into target cells is of a highly variable nature, dependent upon a plurality of factors, *inter alia*, the nature of the antibody, and the epitopic and cellular structural context of the target cell, the art is sufficiently unpredictable to prevent the conclusion that one of ordinary skill in the art would possess a reasonable expectation of success in attaining the presently claimed invention. As such, Examiner's arguments fail to sustain the characterization of the present claims as *prima facie* obvious, and Examiner's rejection to the present claims as obvious under 37 C.F.R. § 103 (a) should be withdrawn.

Rejection Under Obviousness-Type Double Patenting Should be Withdrawn

Claims 59-65, 71, 86 and 93 have been rejected under the judicially created doctrine of obvious-type double patenting as being unpatentable over claims 1-15 of U.S. Patent No. 5,980,896. In response, Applicants intend to submit a Terminal Disclaimer, if appropriate, upon an indication of allowability of the present claims, thereby obviating the double patenting rejection.

CONCLUSION

Applicants respectfully request that the amendments and remarks of the present response be entered and made of record in the instant application. Claims 59-65, 71, 84, and 93-118 fully meet all statutory requirements for patentability. Withdrawal of the Examiner's rejections and allowance and action for issuance are respectfully requested.

Applicant respectfully requests that the Examiner call the undersigned attorney at (212) 790-9090 if any questions or issues remain.

Respectfully submitted,

Date May 6, 2002

Adriane M. Antler 32,605

Adriane M. Antler (Reg. No.)

PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, New York 10036-2711
(212) 790-9090

Enclosures

EXHIBIT A

Marked-up version of the amended claims
Application No. 09/290,708
Dated: May 6, 2002

61. (amended) The immunoconjugate of claim 60 wherein the therapeutic agent is a cytotoxin that is a ribosome binding toxin.

63. (amended) The immunoconjugate of claim 61 wherein the therapeutic agent [ribosome binding toxin] is an exotoxin.

EXHIBIT B

Claims that will be pending in Application No. 09/290,708
as of entry of the instant amendment
Dated: May 6, 2002

59. An immunoconjugate that comprises an antibody joined to a therapeutic agent, wherein the antibody comprises an immunoglobulin or antigen-binding fragment thereof that specifically binds to a Lewis Y cell membrane antigen of a carcinoma cell, wherein upon binding of the antibody to the carcinoma cell, the antibody is capable of being internalized within the cell.

60. The immunoconjugate of claim 59 wherein the therapeutic agent is selected from the group consisting of a cytotoxin, an anti-tumor drug, a radioactive agent, a second antibody, and an enzyme.

61. The immunoconjugate of claim 60 wherein the therapeutic agent is a cytotoxin that is a ribosome binding toxin.

62. The immunoconjugate of claim 61 wherein the ribosome binding toxin is ricin A.

63. The immunoconjugate of claim 61 wherein the therapeutic agent is an exotoxin.

64. The immunoconjugate of claim 63 wherein the exotoxin is Pseudomonas exotoxin A.

65. The immunoconjugate of claim 63 wherein the exotoxin is truncated to remove the cell-binding domain.

71. The immunoconjugate of claim 63 wherein the amino terminus of the exotoxin has been modified to include a lysin amino acid residue.

86. A pharmaceutical composition comprising a pharmaceutically effective amount of the immunoconjugate of claim 59 and a pharmaceutically acceptable carrier.

93. The immunoconjugate of claim 59 wherein the Lewis Y cell membrane antigen comprises a fucosylated variant of a Lewis Y antigen.

94. The immunoconjugate of claim 59, wherein the antibody or antigen-binding fragment thereof is a monoclonal antibody or a fragment of a monoclonal antibody.

95. The immunoconjugate of claim 59, wherein the antibody is a Fab, $F(ab')_2$ or Fv fragment.

96. The immunoconjugate of claim 59, wherein the antibody is a bifunctional antibody with a binding specificity for two different antigens, one of the antigens being said Lewis Y cell membrane antigen of a carcinoma cell.

97. The immunoconjugate of claim 59, wherein the antibody is a chimeric antibody.

98. The immunoconjugate of claim 59, wherein the antibody is a humanized antibody.

99. The immunoconjugate of claim 59, wherein the antibody is a human antibody.

100. The immunoconjugate of claim 59, 94, 97, 98, or 99, which is purified.

101. An immunoconjugate that comprises an antibody joined to a therapeutic agent, wherein the antibody comprises an immunoglobulin or antigen-binding fragment thereof that competitively inhibits binding of the monoclonal antibody BR96, as produced by the

hybridoma deposited with the ATCC and assigned Accession No. HB10036, to a carcinoma cell.

102. The immunoconjugate of claim 101, wherein the antibody is a monoclonal antibody or a fragment of a monoclonal antibody.

103. The immunoconjugate of claim 101, wherein the antibody is a Fab, $F(ab')_2$ or Fv fragment.

104. The immunoconjugate of claim 101, wherein the antibody is a bifunctional antibody with a binding specificity for two different antigens.

105. The immunoconjugate of claim 101, wherein the antibody is a chimeric antibody.

106. The immunoconjugate of claim 101, wherein the antibody is a humanized antibody.

107. The immunoconjugate of claim 101, wherein the antibody is a human antibody.

108. The immunoconjugate of claim 101, 102, 105, 106, or 107, which is purified.

109. The immunoconjugate of claim 101 wherein the therapeutic agent is selected from the group consisting of a cytotoxin, an anti-tumor drug, a radioactive agent, a second antibody, and an enzyme.

110. The immunoconjugate of claim 109, wherein the therapeutic agent is a cytotoxin that is a ribosome binding toxin.

111. The immunoconjugate of claim 110, wherein the ribosome binding toxin is ricin A.

112. The immunoconjugate of claim 110, wherein the therapeutic agent is an exotoxin.

113. The immunoconjugate of claim 112, wherein the exotoxin is Pseudomonas exotoxin A.

114. The immunoconjugate of claim 112, wherein the exotoxin is truncated to remove the cell-binding domain.

115. The immunoconjugate of claim 112, wherein the amino terminus of the exotoxin has been modified to include a lysine amino acid residue.

116. The immunoconjugate of claim 109 which is purified.

117. A pharmaceutical composition, comprising a pharmaceutically effective amount of the immunoconjugate of claim 101, 102, 105, 106, or 107, and a pharmaceutically acceptable carrier.

118. The pharmaceutical composition of claim 117, wherein the immunoconjugate is purified.

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

ECREAL 182 (1) 1–295 (1989
ISSN 0014-482

EXPERIMENTAL RESEARCH

VOLUME 182 · MAY 1989 · NUMBER 1

ACADEMIC PRESS, INC.
Harcourt Brace Jovanovich, Publishers

San Diego New York Boston
London Sydney Tokyo Toronto

Experimental Cell Research 182 (1989) 215–233

Modulation of Transferrin Receptor Expression and Function by Anti-transferrin Receptor Antibodies and Antibody Fragments

JAYNE LESLEY,[1] ROBERTA SCHULTE, and JOHN WOODS*

*Department of Cancer Biology, The Salk Institute, P.O. Box 85800, San Diego, California 92138, and *Yale University School of Medicine, Department of Cell Biology, New Haven, Connecticut 06510*

It has been suggested that effects of anti-transferrin receptor antibodies on cell growth and receptor expression are the result of varying degrees of receptor crosslinking by bi- and multivalet binding agents. In order to study this question directly, we have cultured murine lymphoma cells in mono- and divalent fragments from IgG and IgM monoclonal anti-transferrin receptor antibodies and in intact antibodies. The studies presented here demonstrate that effects of antibody binding on transferrin receptor distribution, metabolism, and function depend, at least in part, on antibody valence, and therefore on the degree of crosslinking of receptors by antibody. We found that monovalent antibody fragments did not significantly alter cell growth, receptor surface expression, intracellular localizaton, or degradation. Diavalent antibody caused a uniform down-regulation of cell-surface receptor expression, which was accompanied by increased degradation only when antibody Fc was present. Normal receptor cycling apparently continued, despite the reduction in surface expression. Culture in multivalent IgM antibody, however, resulted in accumulation of antibody-complexed receptor on the cell surface without internalization and caused profound inhibition of cell growth. Thus, we show two mechanisms by which different degrees of antibody crosslinking can influence transferrin receptor function: by receptor down-regulation and blocking internalization.

The expression of functional transferrin receptors on the cell surface is closely associated with cell growth [1–6]. In growing cells, transferrin receptor cycles continuously from the cell surface to intracellular compartments and back to the cell surface [7–13]. Antibodies against transferrin receptor have been shown to cause a redistribution of surface receptors and in some cases to inhibit cell growth [6, 14–25]. The mechanisms of these antibody effects are of considerable interest since anti-transferrin receptor antibodies are often used to inhibit cellular functions *in vitro* [6, 16, 18–22, 25] and antibodies against cell-surface receptors for essential growth factors (such as transferrin receptor) have been proposed as anti-tumor agents *in vivo* [26–28].

In both murine and human systems, polymeric anti-transferrin receptor antibodies (IgM and IgA) are more effective inhibitors of *in vitro* cell growth than are receptor-specific antibodies of the IgG classes [15, 16, 19, 22, 23]. IgG anti-receptor antibodies become inhibitory when crosslinked with anti-immunoglobin [19, 23]. The growth inhibitory effects are not attributable to competition for the transferrin binding site [19, 23, 25]. Both IgG and IgM anti-transferrin receptor antibodies can cause redistribution of cell-surface receptors [17, 19, 22–24].

[1] To whom reprint requests should be addressed.

0014-4827/89 $03.00

Receptor down-regulation by IgG antibody has been shown to be accompanied by an increased rate of receptor degradation [19, 24].

It has been suggested that whether anti-transferrin receptor antibodies are growth inhibitory or cause receptor down-regulation (here, we use down-regulation to indicate a reduction in receptor levels on the cell surface, not necessarily accompanied by degradation) is determined by the degree of crosslinking of receptors by bi- and multivalent binding agents [19, 23]. In order to study this question further, we have prepared monovalent Fab fragments from an IgM and an IgG_{2a} anti-murine transferrin receptor antibody and divalent $F(ab')_2$ fragments from the IgM antibody. We have studied the effects of monovalent, divalent, and multivalent receptor-binding antibodies on cell growth, receptor cell-surface expression, intracellular localization, and degradation.

MATERIALS AND METHODS

Cells. The AKR/J thymic lymphoma cell line AKR1 was used for all these studies [29]. It was cultured in 10% horse serum in Dulbecco's modified Eagle's medium.

Antibodies. The monoclonal anti-transferrin receptor antibodies RI7 208, a rat IgM; RI7 217, a rat IgG_{2a}; and RL34-14, another rat IgG_{2a}; and their purification from ascites fluid and serum of Balb/c Nu/Nu mice have been described [18, 19]. Purified antibody was used for preparation of fragments, iodination, blocking, growth, and immunolocalization studies.Culture supernatants from the hybridomas producing the antibodies were used for flow cytometry and immunoprecipitations.

Preparation and testing of antibody fragments. Monovalent Fab fragments of purified monoclonal antibodies RI7 208 and RI7 217 were prepared by papain digestion [30] and ion exchange chomatography on GE52 Sephadex (Whatman). Conditions for digestion of each antibody were chosen (on the basis of complete digestion of heavy chain and a good yield of light-chain containing fragments) after trial digestion at differing papain/protein ratios and times of digestion. Complete digestion of the antibody heavy chain was verified by gel electrophoresis (sodium dodecyl sulfate (SDS)[2]-10% polycrylamide reducing gels, Ref. [31]) of the total digest and of the fractions eluted from the GE52 column. In mixing experiments, we could readily detect 5% contamination by intact IgM heavy chain and still lower levels of intact IgG heavy chain. Any undigested material would have been below these levels. Column fractions were screened for antibody activity and the absence of Fc determinants by flow cytometric analysis as follows: column fractions were adjusted to 0.035 OD_{280} units/ml in PBS. One hundred microliters of this dilution of each column fraction was incubated with two aliquots of 10^6 AKR1 cells for 30 min at 4°C. Following two washes in the cold, one aliquot was incubated (30 min at 4°C) with flurescein-labeled anti-rat Ig specific for heavy and light chain (Cappell Laboratories) and the other with fluorescein-labeled anti-rat specific for the appropriate heavy chain Fc: anti-IgM (Hyclone Laboratories) or anti-IgG (Cappell Laboratories). (Specificity of the latter reagents for their respective Fc determinants was titrated using intact RI7 217 (IgG_{2a}) and RI7 208 (IgM) antibodies bound to AKR1 cells. The fluorescent-labeled, Fc-specific anti-IgG was used at a dilution such that it labeled cells preincubated with RI7 217, but did not label cells preincubated with RI7 208. Similarly, the anti-IgM antibody stained cells preincubated with RI7 208 but not with RI7 217.) After two more washes in the cold, the samples were run on a modified Los Alamos flow microfluorimeter as described previously [32]. Column fractions showing anti-transferrin receptor activity without binding the anti-Fc-specific reagent and containing light chain but no detactable intact heavy chain on SDS–acrylamide gels were pooled.

A divalent $F(ab')_2$ fragment of RI7 208 (IgM) antibody was prepared by pepsin digestion (2 h at 37°C with a pepsin/protein ratio (w/w) of 2%, Ref. [33]) and separation on Sephacryl-S200 (Pharmacia). Column fractions were screened on SDS–acrylamide gels (reducing conditions) for the absence of intact heavy chain and the presence of light chain, and for the presence of a band around 100,000 M_r in nonreducing gels. These fractions were also screened for binding to cells by flow cytometry as described above. Pepsin digestion of RI7 217 was also attempted in order to produce a divalent $F(ab')_2$

[2] Abbreviations used: PBS, phoshate-buffered saline, 0.15 *M* NaCl, 10n*M* NaH_2PO_4 (pH 7.2); OVA, ovalbumin; SDS, sodium dodecyl sulfate.

fragment of the IgG_{2a}. This digestion was neither efficient nor uniform. Undigested, intact heavy chain remained along with heterogeneous fragments of heavy chain. This resulted in a mixture of intact antibody and various fragments including a monovalent Fab with an M_r around 50 kDa. Though there appeared to be a diavalent $F(ab')_2$ fragment in this digest, the yield was very low and it could not be separated reliably from the other components.

Blocking of ^{125}I-antibody binding by fragments. Transferrin receptor binding activity of Fab preparations was determined by titrating their ability to block the binding of ^{125}I-labeled intact RI7 208 or RI7 217 to AKR1 cells as described previously [18, 34]. Serial twofold dilutions of unlabeled fragment preparations were added to cells in microwells, followed by the addition of a subsaturating concentration of ^{125}I-labeled intact antibody. Blocking curves for the antibody fragments were compared to curves for intact unlabeled antibody.

In growth, modulation, degradation, and microscopy studies, the concentration of the antibody fagment preparations was adjusted to give binding equivalent to that of intact antibody.

Cell growth. Cell growth in the presence of antibody fragments was determined as previously described ([18] and legend to Fig. 3).

Quantitation of cell-surface receptors. Flow microfluorimetry was used to quantitate levels of surface receptor expressed on AKR1 cells cultured in anti-transferrin receptor antibodies and fragment preparations. Staining, washes, and analysis on modified Los Alamos flow microfluorimeter were performed as described previously [32]. Because of differences in the staining levels seen with the different antibody preparations, all cells were restained with a third anti-transferrin receptor antibody (RL34-14) in order to maximize labeling of cell-surface receptors. (The differences in staining intensity among the antibodies appear to be due to differences in the affinity of the fluorescein anti-rat Ig second reagent for IgG, IgM, and Fab antibodies.) RL34 binds to a different determinant than RI7 208 or RI7 217 [19, 34] and binds the polyspecific Fl anti-rat Ig well. Though RI7 208 and RI7 217 compete with RL34 binding, due to the close proximity of all the binding sites [19], the binding of RL34 in combination with antibodies or fragments on the cell surface gives a measure of the total surface receptor when stained with polyspecific Fl anti-rat Ig (Cappell Laboratories). In fact, staining with several different anti-receptor antibodies gave the same results.

Receptor degradation. To study the fate of the surface transferrin receptor in the presence of anti-transferrin receptor antibodies, AKR1 cells were surface ^{125}I-iodinated under sterile conditions with lactoperoxidase [31]. Washed cells were then put into several replicate dishes in the presence or absence of 10 μg/ml of RI7 217 antibody or the equivalent concentration of antibody fragment. Antibody was added at 4°C and allowed to bind to the cells before culture at 37°C. For the 0 time point, cells were harvested without warming to 37°C. At various times after the initiation of culture, cells were washed in phosphate-buffered saline [PBS, 0.15 *M* NaCl, 10 m*M* NaH_2PO_4 (pH 7.2)] and solubilized in 1% Nonidet-P40 in PBS. Nuclei were spun out (5 min at 850*g*), and the lysates were precipitated with hybridoma supernatants and polyspecific anti-rat immunoglobulin [35]. Precipitates were reduced and run on SDS–10% polyacrylamide gels.

Immunolocalization. Cells used for immunolocalization experiments were harvested from experimentally growing cultures at densities of $5–10\times10^5$ cells/ml. For fluorescent light microscopy experiments, cells were fixed for 30 min by adding an equal volume of 4% formaldehyde (freshly prepared from paraformaldehyde) in PBS directly to the culture medium. For immunoperoxidase electron microscopy experiments, cells were fixed overnight by adding an equal volume of fixative prepared in a 2× solution such that the final concentrations were 2% formaldehyde, 0.075 *M* lysine, 0.01 *M* $NaIO_4$, and 0.0375 *M* $NaPO_4$, pH 6.2. In some experiments, anti-transferrin receptor antibody preparations (RI7 217, 10 μg/ml; Fab from RI7 217, 20 μg/ml; RI7 208, 10 μg/ml; $F(ab')_2$ from RI7 208, 20 μg/ml) were added to the culture media and incubated for 5–60 min at 37°C prior to fixation. After fixation, cells were washed three times (by centrifugation and resuspension) in protein-free PBS. The final pellet was resuspended in a minimal volume of PBS, and a 15-μl aliquot containing $10^5–10^6$ cells was placed on a polylysine-coated glass microscope slide [36]. The cells were allowed to adhere for 30 min after which the slides were extensively washed with PBS containing 0.1% ovalbumin (PBS/OVA) to remove unbound cells, leaving an attached closely spaced monolayer.

For immunofluorescent observation, attached cells were permeabilized by incubation with 0.1% Triton X-100 in PBS for 10 min followed by washing with PBS/OVA. Labeling of attached, permeabilized cells was carried out by placing a 40-μl droplet of the appropriate reagent diluted in PBS/OVA over the cells. For visualization of transferrin receptors, cells were first incubated with rat anti-mouse transferrin receptor antibody (10–20 μg/ml) followed by incubation of fluorescein-conjugated rabbit anti-rat Ig (Cappel Laboratories, diluted 1:50). For visualization of prebound anti-transferrin receptor, the cells were incubated only with the latter reagent. Generally the cells were incubated in each antibody for 1–2 h, rinsed (five times) with PBS/OVA after each incubation, mounted in phenylenedia-

mine/glycerol [37], and examined in a Zeiss photomicroscope III equipped with epifluorescence illumination. Photomicrographs were taken with the largest diameter of the cells in the plane of focus in order to clearly distinuish intracellular label from surface label. All fluorescent micrographs were taken at an exposure of one minute and all prints within a single figure were exposed and processed for the same times in order to facilitate comparisons between signals obtained with different antibody preparations.

For electron microscopic observation of the distribution of prebound anti-transferrin receptor antibodies, the immunoperoxidase procedure previously described was employed [13]. In brief, the fixed attached cells were incubated in a 20-μl droplet of PBS/OVA containing 0.05% saponin (PBS/OVA/SAP). The cells were then incubated for 90 min with a rabbit anti-rat IgG (1 : 50 dilution) followed by horseradish peroxidase-conjugated sheep anti-rabbit Fab (1 : 100 dilution) (Biosys, Compiegne, France) for 1–2 h each. After each antibody incubation the cells were washed with five to seven changes of PBS/OVA/SAP. The cells were then fixed for 30 min in 1.5% gluteraldehyde in 100 m*M* sodium cacodylate, pH 7.4, containing 5% sucrose, followed by extensive washing with 50 m*M* Tris containing 7.5% sucrose. The perioxidase reaction was initiated by covering the cells with 50 μl of a mixture of 1 ml of 0.2% diaminobenzidine in 50 m*M* Tris, pH 7.4, containing 7.5% sucrose plus 15 μl of 0.3% H_2O_2 (final H_2O_2 concentration 0.005%) and allowed to proceed for 5–15 min (until the cells appeared brown under a dissecting microscope). The reaction was terminated by extensive washing with 50 m*M* Tris, pH 7.4, containing 7.5% sucrose. The cells were postfixed in a 50-μl drop of ferrocyanide-reduced OsO_4 for 20 min at room temperature. Dehydration and embedding in Epox 812 were carried out on the slide and, after the final change of Epox, the slide was placed upside down on a silicon rubber embedding mold and polymerized overnight at 60°C. The cells thus embedded were separated from the glass with a razor blade, and selected areas (~1 mm^2) were mounted on a support block of Epox and sectioned *en face*. Sections were stained with lead citrate and examined in a Philips 301 electron microscope operated at 60 kV.

RESULTS

Antibody Fragments

Figure 1 shows the results of SDS–polyacrylamide gel electrophoresis of intact RI7 208 and RI7 217 monoclonal antibodies and their purified fragments under reducing and nonreducing conditions. Intact RI7 208, an IgM antibody [18], does not enter the (7.5% acrylamide) nonreduced gel. Reduced and run on a 10% acrylamide gel, it has a light chain of about 30 kDa and a heavy chain of about 70 kDa. The monovalent Fab preparation of RI7 208, nonreduced, shows a major band at about 45 kDa and a minor band at about 60kDa. Reduced, this preparatuin shows a light chain at 30 kDa and several heterogeneous lower molecular weight bands. The divalent $F(ab')_2$ (pepsin digested) preparation of RI7 208 has an unreduced apparent molecular weight of 155 kDa. Reduced, it shows intact light chain at 30 kDa and a fragment of the heavy chain with an apparent molecular weight of 47 kDa. The apparent molecular weight of the unreduced fragment is consistent with a disulfide bonded dimer consisting of two light chains and two heavy chain fragments. Intact, RI7 217, an IgG_{2a} [18], has an apparent molecular weight of 210 kDa unreduced in these gels. Reduced it shows a 32-kDa light chain and 49-kDa heavy chain. The monovalent Fab preparation has two bands unreduced at 54 and 39 kDa, and reduced it contains light chain and several lower M_r bands. Several different preparations of each Fab fragment were made and all gave results similar to those shown in Fig. 1.

Both RI7 208 and RI7 217 monovalent Fab preparations had two major bands on unreduced SDS–acrylamide gels wich migrated at about 54–60 and 39–45 kDa. In two-dimensional electrophoresis, run nonreduced in the first dimension and

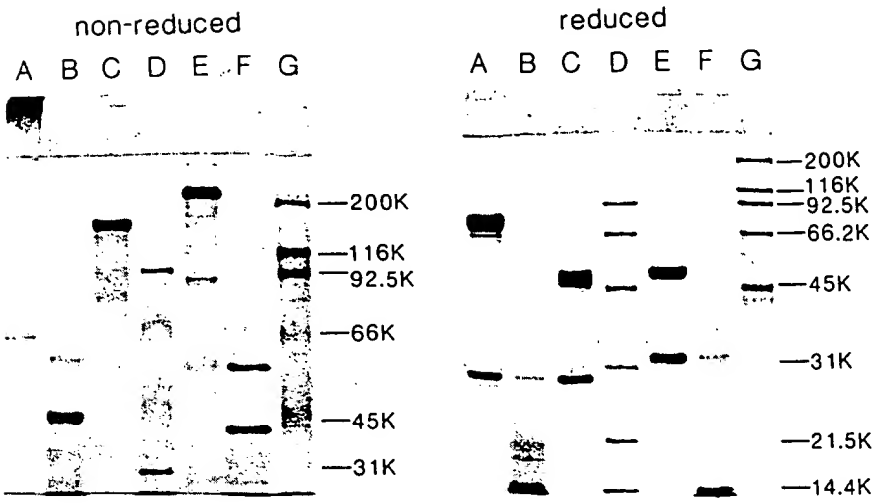


Fig. 1. SDS–acrylamide gel electrophoresis of antibodies and fragments. Two micrograms of purified antibody preparation was loaded in each well. Nonreduced samples were diluted and boiled in sample buffer without mercaptoethanol and run on 7.5 % acrylamide gels. Reduced samples included 0.5 *M* mercaptoethanol in the sample buffer and were run on 10% acrylamide gels. Lanes contained intact RI7 208 IgM antibody (*A*), purified by dialysis against low salt; monovalent Fab of RI7 208 (*B*), prepared by papain digestion and purified by ion exchange chromatography on GE52 (Whatman); divalent F(ab')$_2$ of RI7 208 (*C*), prepared by pepsin digesion and purified by gel filtration on Sephacryl S-200, superfine (Pharmacia); "low"-molecular-weight standards (Bio-Rad) (*D*); intact RI7 217 IgG antibody (*E*), purified on GE52; monovalent Fab of RI7 217 (*F*), papain digest purified on GE52; and high-molecular-weight standards (Bio-Rad) (*G*).

reduced in the second dimension, both bands could be shown to contain antibody light chain. In some experiments, the two bands were partially separated on GE52 Sephadex purification and assayed separately for anti-transferrin receptor activity. Both had activity in blocking the binding of ^{125}I-labeled antibody and both bound to cells as detected by flourescein-labeled anti-rat Ig with heavy- and light-chain specificity, but not detected by Fc specific anti-IgG (see Materials and Methods). Papain has apparently cut the heavy chains of both monoclonal antibodies at several different sites resulting in multiple heavy chain fragments with M_r of below 30 kDa. Some of these fragments remain associated through intrachain disulfide bonds and are able to combine with light chain to give two major species that bind antigen. Despite their molecular complexity, on the basis of their binding to cells and blocking intact antibody, both Fab species from each of the monoclonal antibodies have antigen binding sites and, on the basis of their molecular weights, are monovalent.

Antigen-Binding Activity of Antibody Fragments

Transferrin receptor binding activity of Fab and F(ab')$_2$ preparations was determined relative to intact antibody by titrating the ability of fragments and whole antibody to block binding of ^{125}I-labeled intact RI7 208 or RI7 217 to AKR1 cells. This was done to verify the antigen specificity of the antibody fragments and to adjust their concentration to achieve binding comparable to intact antibody. Exemples of these titrations are shown in Fig. 2. For several different

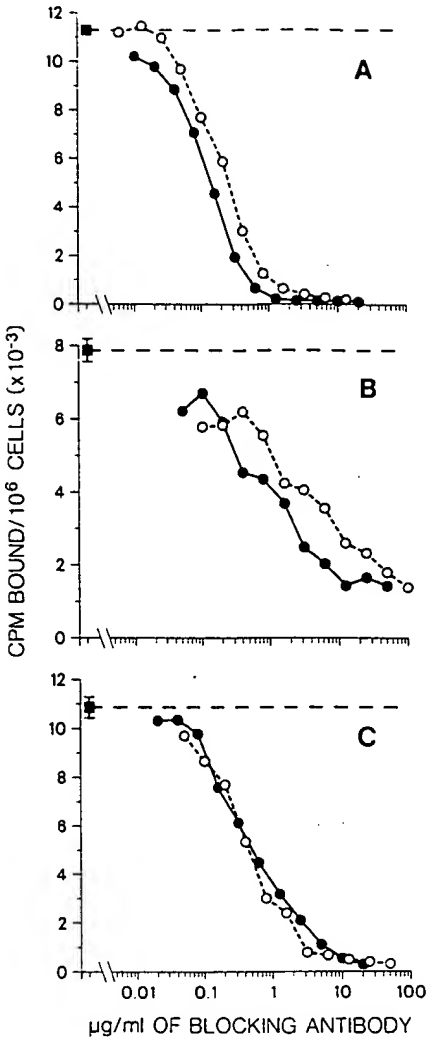


Fig. 2. Blocking of ^{125}I-antibody binding by unlabeled antibody and fragments. ^{125}I-Labeled intact RI7 208 and RI7 217 antibodies were incubated with AKR1 cells in the presence of the indicated concentrations of unlabeled intact antibody (solid circles) or antibody fragments (open circles). Binding of ^{125}I-labeled antibody in the absence of competing cold antibody is indicated by the solid square in each panel. (*A*) ^{125}I-RI7 217 binding in the presence of intact IgG RI7 217 (●—●) and monovalent RI7 217-Fab (○--○). (*B*) ^{125}I-RI7 208 binding in the presence of intact IgM RI7 208 (●—●) or monovalent RI7 208-Fab (○--○). (*C*) ^{125}I-RI7 208 binding in the presence of intact IgM RI7 208 (●—●) or divalent RI7 208-F(ab')$_2$ (○--○). ^{125}I-Labeled antibodies were used at a concentration below the saturation level for the particular preparation and such that sufficient cpm were bound. Different ^{125}I-RI7 208 preparations were used in experiments shown in (*B*) and (*C*).

preparations of RI7 217 Fab, the monovalent preparations gave half-maximal blocking at about 0.5, 1, and 2 times the OD_{280} of whole, unlabeled RI7 217 antibody. Monovalent RI7 208 Fab preparations gave half-maximal blocking at about 2, 3, and 5 times the OD_{280} of whole RI7 208 antibody; and diavalent F(ab')$_2$ from RI7 208 blocked at equal protein concentrations to whole RI7 208.

A pure Fab preparation from RI7 217 would be expected to contain an equivalent number of antigen-binding determinants at about half to two-thirds the protein concentration of intact antibody. A pure Fab from RI7 208 of the sizes we obtained should contain an equal number of binding determinants at about half

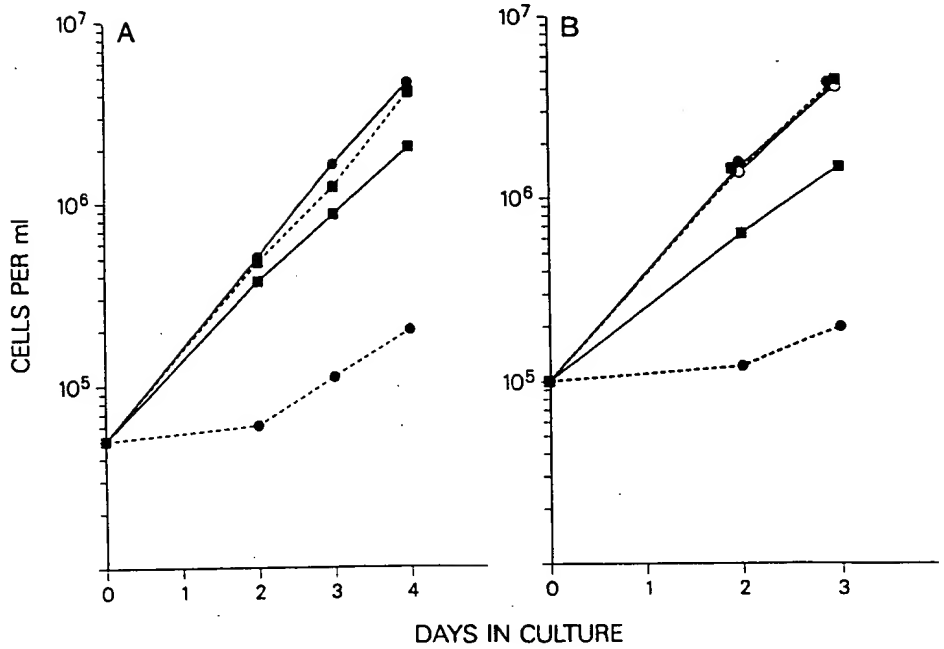


Fig. 3. Growth of AKR1 cells in the presence of anti-transferrin antibodies and fragments. AKR1 cells were plated in 35-mm petri dishes at 5×10^4 cells or 1×10^5 cells/ml. Intact antibody at 10 μg/ml or antibody fragments adjusted to a dilution equivalent in binding activity to 10 μg/ml of whole antibody (see Materials and Methods) was added. Cells were counted at the indicated times on a Coulter counter. (*A*), AKR1 cells without antibody (●—●); 10 μg/ml intact IgM RI7 208 (●--●); 30 μg/ml RI7 208 monovalent Fab (■--■); 10 μg/ml intact IgG RI7 217 (■—■). (*B*), AKR1 cells without antibody (●—●), 10 μg/ml intact IgM RI7 208 (●--●); 10 μg/ml RI7 208 divalent F(ab')$_2$ (■--■); 10 μg/ml intact IgG RI7 217 (■—■); 20 μg/ml RI7 217 monovalent Fab (○--○).

the protein concentration of intact RI7 208, and the RI7 208 (Fab')$_2$ should bind equally at about two-thirds the concentration of whole antibody. The reduced binding activity of some of the Fab preparations relative to intact antibody could be due to (1) reduced avidity because of the absence of the stabilizing contribution of multivalent binding, and/or (2) the presence of inactive protein fragments in the preparations. Since some of the RI7 217 preparations gave binding equivalent to intact antibody, we believe the reduced activity of some preparation was due to the presence of inactive protein fragments in these RI7 217 Fabs. Both factors may contribute to the relatively poor blocking activity of the RI7 208 Fab preparations. In growth, modulation, degradation, and microscopy studies, the concentration of the antibody fragment preparations was adjusted to give binding equivalent to that of intact antibody.

Cell Growth in the Presence of Antibody and Fragments

The IgM antibody RI7 208 inhibits cell growth (>90% inhibition) and reduces cloning efficiency of AKR1 lymphoma cell lines to about 10^{-5}, while cells grown in IgG antibodies such as RI7 217 show about 50% reduction in growth rate and minimal effects on cloning efficiency [19]. We cultured AKR1 cells in monovalent Fab fragments from the IgM antibody and assayed cell growth up to 4 days. Figure 3*A* shows that Fab fragments of RI7 208 had no effect on cell growth. The

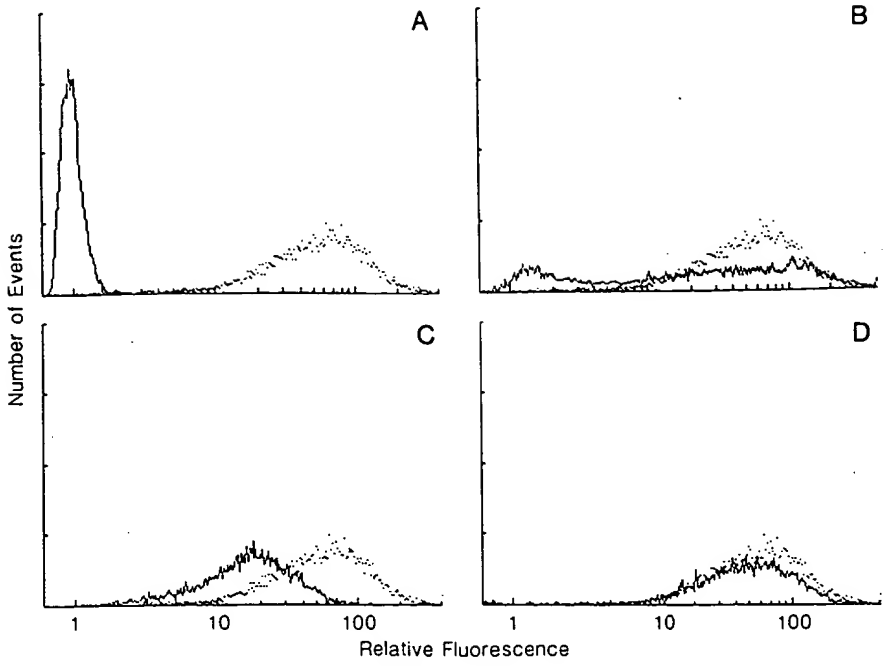


Fig. 4. Redistribution of cell-surface transferrin receptor in the presence of antibodies and fragments. AKR1 cells were plated at 2×10^5 cells/ml in the presence of intact antibody at 10 μg/ml or an equivalent dilution of RI7 217 monovalent Fab (10 μg/ml). The next day, cells were chilled, harvested, and kept cold during subsequent washes and incubations. For flow microfluoremetry, cells were stained with no antibody or anti-transferrin receptor antibody RL34-14 hybridoma supernatant, followed by fluorescein labeled anti-rat immunoglobulin. Dotted lines in each panel indicate control cells (cultured in the absence of antibody) stained with RL34-14 anti-transferrin receptor (positive control). (*A*) Control cells stained with fluorescein anti-rat Ig only (negative control); (*B*) cells cultured overnight in intact RI7 208 IgM antibody; (*C*) cells cultured overnight in intact RI7 217 IgG antibody; (*D*) cells cultured overnight in monovalent RI7 217-Fab. Cells in (*B*), (*C*) and (*D*) were restained with RL34-14 before addition of fluorescein-labeled anti-rat Ig (heavy and light chain poly-specific). The geometric mean fluorescence of the background stained population (*A*) was set at 1.0 units. Relative to this background, the fluorescence of the positive population (dotted insert) was 42.9 units; of (*C*), 13.6 units; of (*D*), 35.0 units. The cells in (*B*) were too heterogeneous for such a measure to be meaningful.

intact RI7 208 antibody caused 96% inhibition of cell growth (in three experiments 95 to 98% inhibition) at Day 4. The IgG_{2a} antibody RI7 217 reduced cell growth by about 55% at 4 days (40 to 60% in three experiments). Growth of cells cultured in monovalent Fab from RI7 208 was equal to controls on the absence of antibody. Flow cytometric analysis of the cells cultured in the RI7 208 Fab antibody using a flourescent polyspecific anti-rat Ig showed the presence of the Fab on the cell surface (not shown).

In Fig. 3*B*, AKR1 cells were cultured 3 days in the presence of divalent RI7 208 $F(ab')_2$ and monovalent RI7 217 Fab, as well as in intact RI7 208 and RI7 217. Only the multivalent RI7 208 antibody inhibited cell growth significantly (>90% inhibition). Intact RI7 217 again reduced cell growth to about 50% of the control value at 3 days.

Modulation of Cell-Surface Receptor by Anti-Transferrin Receptor Antibodies

To study the cell-surface expression of transferrin receptor, AKR1 cells cultured in the presence of antibodies and fragments of different valences were

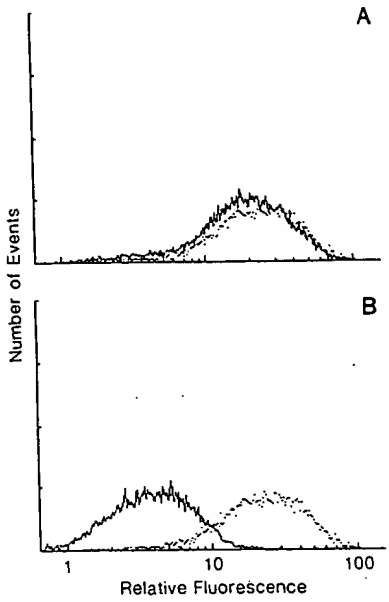


Fig. 5. Redistribution of cell-surface transferrin receptor in the presence of RI7 208 antibody fragments. AKR1 cells were cultured as described in Fig. 4 in the presence of (*A*) monovalent RI7 208-Fab (30 μg/ml) or (*B*) divalent RI7 208-F(ab')$_2$ (10 μg/ml), and stained with RL34-14 IgG anti-transferrin receptor supernatant and fluorescein anti-rat Ig (polyspecific) as described in Fig. 4. The dotted line in each panel indicates control cells (cultured without added antibody) stained with RL34-14 anti-transferrin receptor (geometric mean fluorescence of 21.2 unites, relative to unstained cells set at 1.0 units).The relative fluorescence of (*A*) was 17.0 units; of (*B*) 3.9 units.

stained with anti-receptor antibody and fluoresceinated anti-rat immunoglobulin (polyspecific), and analyzed by flow microfluorimetry.

Figure 4 shows the levels of transferrin receptor observed on control cells (dotted insert) and cells cultured overnight in RI7 217 (Fig. 4*C*), RI7 208 (Fig. 4*B*), and monovalent RI7 217-Fab (Fig. 4*D*) relative to background staining with fluorescent anti-Ig alone (Fig. 4*A*). As we observed previously [19], overnight culture in RI7 208 (Fig. 4*B*) results in extremely variable levels of transferrin receptor on AKR1 cells. While some cells have reduced their receptor to the level of background staining, most still express receptor at varying levels, some at higher then control levels (also see [19]). Culture in RI7 217 (Fig. 4*C*), however, results in a uniform down-regulation of cell-surface receptor (three to fourfold in this experiment) when compared to receptor expression in cells cultured without added antibody (dotted curve). In contrast to the down-regulation seen with intact antibody, cells cultured overnight in monovalent RI7 217-Fab show little change in cell-surface receptor expression. Similar results were seen after culture for 2 days in these antibody preparations: down-regulation in intact RI7 217, no change in RI7 217-Fab, variable expression in RI7 208.

Figure 5 shows another experiment in which AKR1 cells were cultured overnight in monovalent Fab and diavalent, F(ab')$_2$ fragments of RI7 208 antibody. Culture in RI7 208-Fab (Fig. 5*A*) had little effect on levels of transferrin receptor expression while divalent F(ab')$_2$ caused uniform fivefold down-regulation of cell-surface receptor. In the same experiment, culture in intact RI7 217 antibody

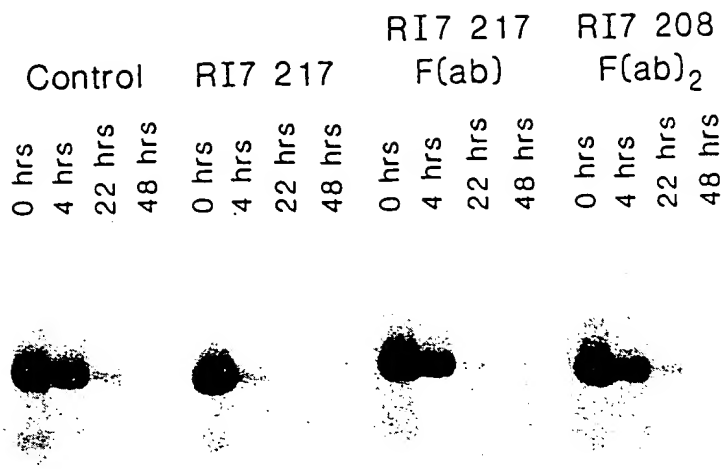


Fig. 6. Degradation of cell-surface iodinated transferrin receptor in the presence of anti-receptor antibody. AKR1 cells were ^{125}I-surface labeled with lactoperoxidase. Equal aliquots of labeled cells were cultured in medium only (Control), in intact RI7 217 IgG antibody (10 μg/ml), in monovalent Fab fragment of RI7 217 (20 μg/ml) or in divalent F(ab')$_2$ RI7 208 (10 μg/ml) for the indicated times. 0 time cultures were exposed to antibody at 4°C and harvested without warming to 37°C. Harvested cells were solubilized in 1% Nonidet-P40 and the membranes were immunoprecipitated with RI7 208 IgM antibody and polyspecific anti-rat immunoglobulin. Equal aliquots of the precipitates were loaded on 10% SDS–polyacrylamide gels for electrophoresis.

resulted in a seven- to eightfold down-regulation. Again, the results after 2 days in culture with the antibody preparations were similar to those seen at 1 day.

Degradation of Cell-Surface Receptor in the Presence of Intact RI7 217 and Fab

We have previously shown that down-regulation of cell-surface receptor during culture in RI7 217 antibody is accompanied by an increase in the rate of degradatoin of surface receptors labeled with ^{125}I by lactoperoxidase [19]. Figure 6 shows this accelerated degradation upon culture in intact RI7 217 but not in cells cultured in the presence of the monovalent fragment of RI7 217 antibody or in the presence of divalent F(ab')$_2$ from RI7 208. The latter result was unexpected, as we predicted that the divalent F(ab')$_2$ would mimic the effects of intact RI7 217. Also, no increase in degradation was seen in cultures in the presence of monovalent Fab from RI7 208 (not shown) or intact RI7 208 (Ref. [19]).

Distibution of Transferrin Receptors in Cells Fixed at Steady State

Figure 7 demonstrates the normal steady-state distribution of transferrin receptors in AKR1 hybridoma cells. Untreated cells were fixed and permeabilized, and transferrin receptors were visualized using RI7 217 antibody and fluorescein-conjugated anti-rat Ig. As expected from our previous studies on transferrin receptor distribution in a plasmacytoma cell line [13], cells labeled with intact RI7 217 (IgG) developed a finely punctate surface label as well as a single large juxtanuclear spot (Fig. 7*a*). Background staining with fluorescein anti-rat Ig is shown in Fig. 7*b*.

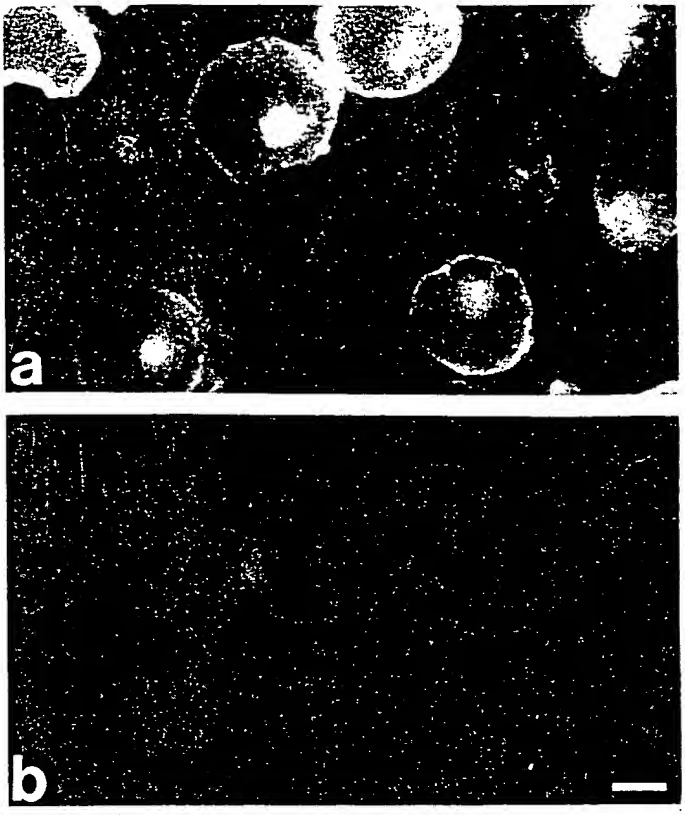


Fig. 7. Indirect immunofluorescence localization of transferrin receptors in cultured AKR1 cells (steady state). Fixed, untreated cells were permeabilized and incubated with (*a*) intact IgG RI7 217 or (*b*) buffer only, followed by fluorescein-conjugated rabbit anti-rat Ig. Bar, 20 μ*M*.

Comparison of the Distribution of Bound Transferrin Receptor Antibodies and Fragments after Uptake at 37°C

In order to follow the intracellular fate of transferrin receptors which have bound antibody at the cell surface, we carried out a series of experiments in which cells were incubated in the presence of antibodies or antibody fragments at 37°C for 5–60 min followed by fixation and immunolocalization of the bound reagent. Cells incubated with divalent RI7 217 IgG (Figs. 8 *a–c*), monovalent Fab fragments derived from RI7 217 (Figs. 8 *d–f*), or divalent $F(ab')_2$ fragments derived from RI7 208 (Figs. 9 *d–f*) all yielded similar results. After 5, 30, or 60 min of incubation at 37°C in the presence of the antibody, a significant proportion of the antibody had been internalized into a single juxtanuclear spot.

However, there were subtle differences in the plasma membrane labeling obtained with each antibody preparation. After 5 min of incubation, the intact RI7 217 IgG displayed a finely punctate pattern along the plasma membrane which after 30 or 60 min became somewhat granular (suggesting aggregation of receptors) and was much less intense (possibly demonstrating a down-regulation of the number of surface receptors) (Figs. 8 *a–c*). The divalent $F(ab')_2$ fragment of RI7 208 IgM displayed a similar pattern on the plasma membrane, except it was granular after only 5 min and did not noticeably decrease in intensity at 30 and 60

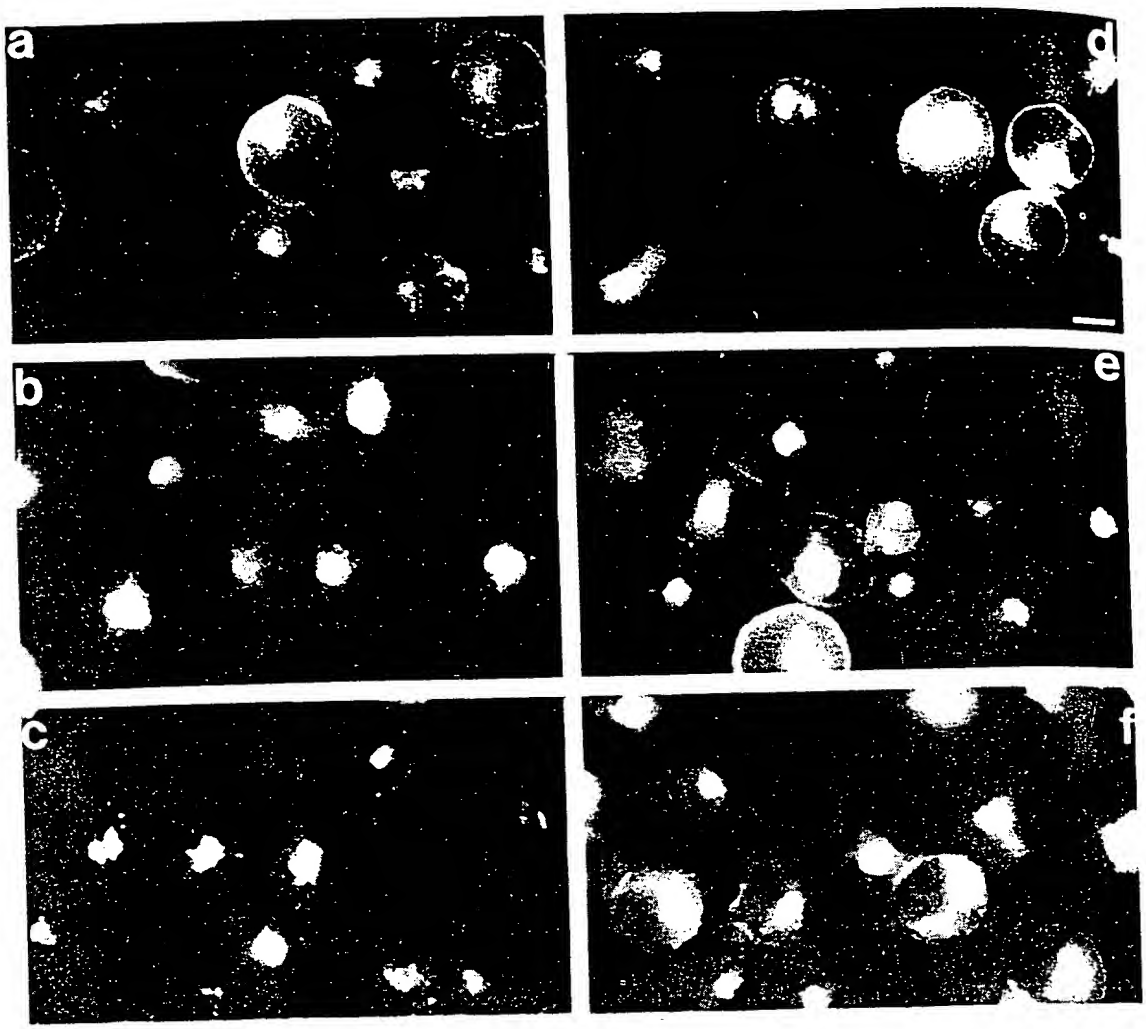


Fig. 8. Immunofluorescence localization of RI7 217 IgG and its Fab fragment. AKR1 cells were cultured in intact anti-transferrin receptor antibody RI7 217 (*a, b, c*) and monovalent Fab fragment of RI7 217 (*d, e, f*) at 37°C for 5 min (*a, d*), 30 min (*b,e*) or 60 min (*c, f*), and then fixed, permeabilized, and incubated with fluoresceinated rabbit anti-rat Ig to visualize the distribution of bound antibodies. In every case, greater than 95% of the cells observed had internalized the anti-transferrin receptor antibody probe into a single juxtanuclear spot. Bound antibodies were also observed in punctate distribution along the cell surface. Bar, 20 μ*M*.

min (Figs. 9 *d–f*). The monovalent Fab fragments of RI7 217 displayed a finely punctate plasma membrane pattern at all times (Figs. 8 *d–f*). At 0 time, all the reagents were found only at the cell surface in a finely punctate pattern (not shown).

In contrast, cells incubated with intact RI7 208 IgM appeared very different (Figs. 9 *a–c*). After 5 min at 37°C the bound antibody was distributed in a coarse granular pattern around the cell periphery. After 30 min the antibody appeared to be sequestered into a single large patch or cap on the cell surface. After 60 min most of the cells still looked the same as after 30 min but approximately 20% appeared to have lost their caps and displayed no apparent binding of RI7 208. At no point was there any evidence that RI7 208 was internalized by AKR1 cells.

In order to verify and extend these observations to the electron microscopic

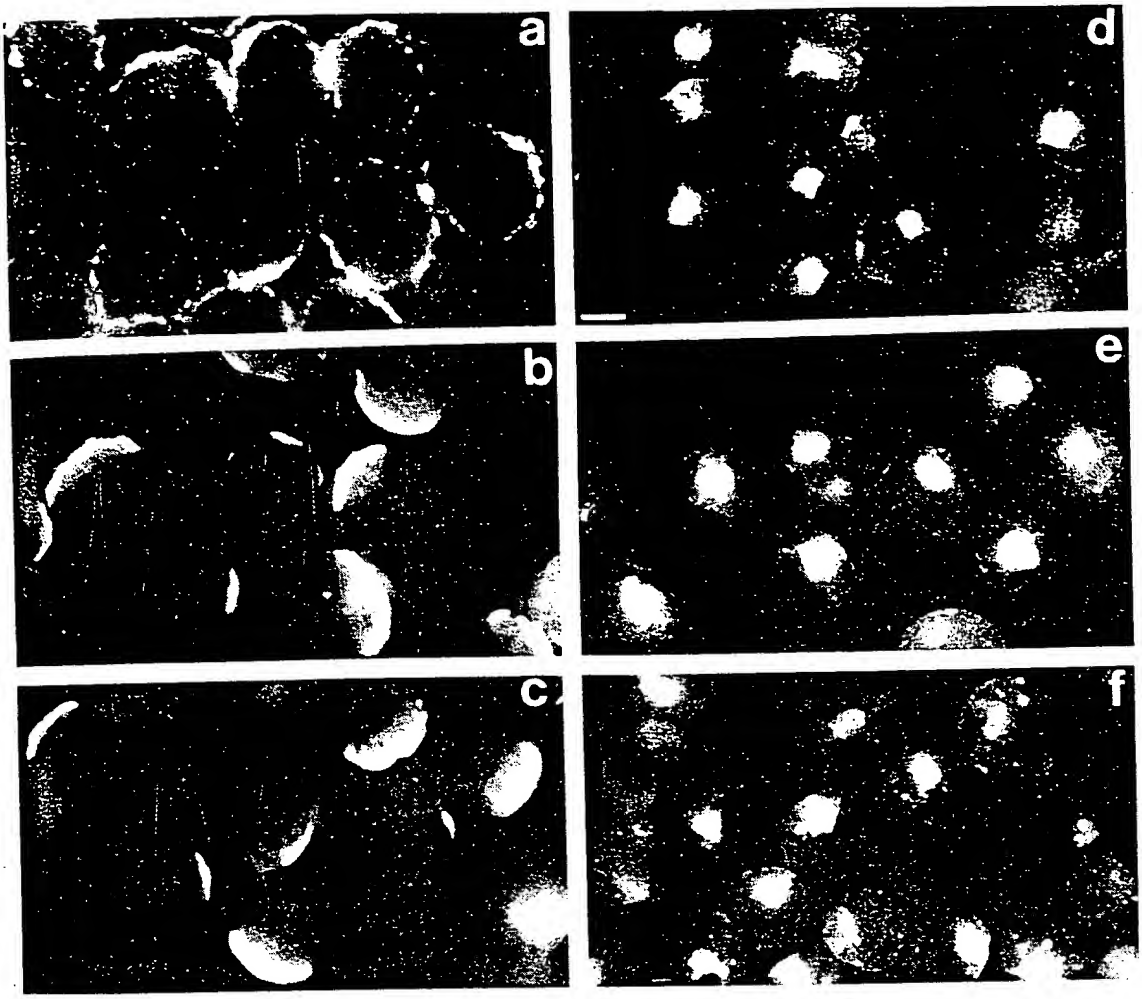
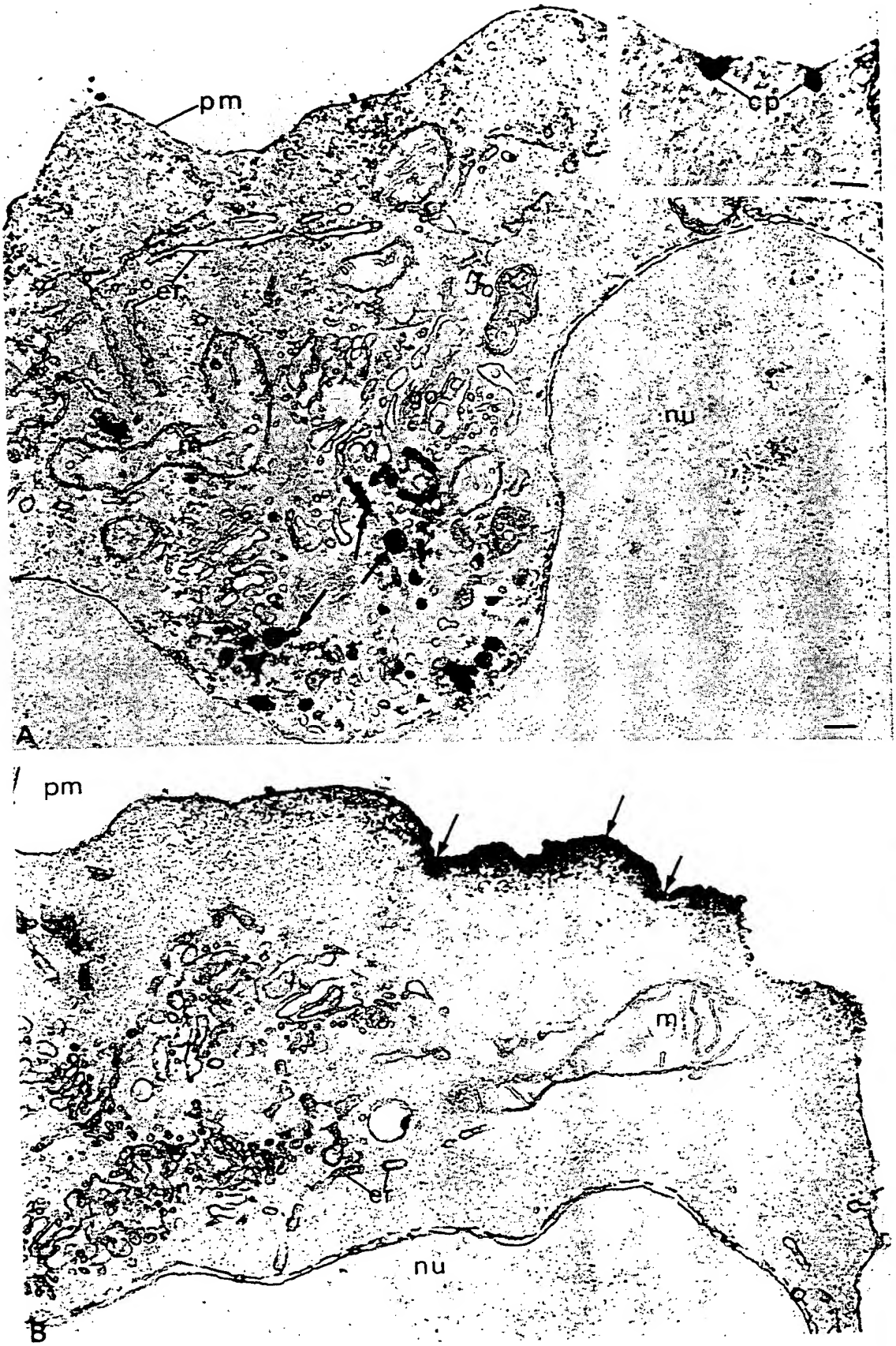


Fig. 9. Immunofluorescence localization of RI7 208 IgM antibody and its divalent $F(ab')_2$ fragment. Cells were incubated with either intact RI7 208 IgM (*a, b, c*) or $F(ab')_2$ fragments of RI7 208 (*d, e, f*) at 37°C for 5 min (*a, d*), 30 min (*b, e*), or 60 min (*c, f*) and than fixed, permeabilized, and incubated with FITC rabbit anti-rat Ig to visualize the distribution of bound antibody. After 5 min at 37°C in the presence of intact RI7 208, the antibody was observed bound along the cell surface in a coarse granular pattern that was uniformly distributed around the cell perihpery (*a*). After 30 min at 37°C the bound IgM appeared to be sequestered into a single large patch or cap on the cell surface (*b*). After 60 min at 37°C the caps appeared to be somewhat smaller and approximately 20% of the cells appeared to have lost their caps (*c*). At no time was there any suggestion that the bound antibody was internalized into the cells. In contrast, after 5 min at 37°C in the presence of divalent $F(ab')_2$ fragments, the antibody probe was observed in a finely punctate distribution around the cell periphery and also on a single intracellular juxtanuclear spot (*d*). Very similar patterns of antibody distribution were observed in cells fixed after 30 min (*e*) or 60 min (*f*) of incubation in the presence of $F(ab')_2$ fragments. Bar, 20 μM.

level, the distribution of prebound RI7 217 or RI7 208 was observed using an immunoperoxidase technique (Fig.10). In cells incubated in the presence of RI7 217 (Fig. 10*A*) for 60 min prior to fixation, immunoreactive RI7 217 was observed in coated pits along the plasma membrane and in intracellular enclosed structures which were found in both the peripheral cytoplasm and associated with the Golgi apparatus, but not within stacked Golgi cisternae. In contrast, cells incubated in the presence of RI7 208 for 60 min (Fig. 10*B*) prior to fixation displayed immuno-


pm
cp
er
nu
A
pm
m
er
nu
B

reactive antibody only along the plasma membrane. These immunoreactive antibodies were not restricted to coated pits as was the case with RI7 217, but appeared to cover entire stretches of the plasma membrane up to several hundred nanometers in length. Also in contrast to RI7 217, RI7 208 was only very rarely observed to be internalized into any intracellular structures.

DISCUSSION

The aim of these studies has been to investigate the role of receptor crosslinking in the effects of anti-transferrin receptor antibodies detected on cells *in vitro*. Anti-transferrin receptor antibodies have been shown to alter cell-surface expression of receptor, receptor degradation, and cell growth and survival [6, 14–25]. The dramatic effects of multivalent anti-transferrin receptor antibodies on growth rate and cloning efficiency [15, 16, 18, 19, 22, 23] suggested that crosslinking may be involved in these inhibitory effects. This idea was further supported by findings that growth inhibiting antibodies did not compete for the transferrin binding site and that noninhibitory IgG antibodies could be made growth inhibitory by crosslinking with anti-immunoglobulin [19, 23].

Mutant lymphoma lines, selected for the ability to grow unimpaired in RI7 208 (IgM) antibody [18, 34], provide further support for the idea that extensive antibody crosslinking of receptors is a key factor in growth inhibition. These cells have RI7 208 binding sites on only half of their transferrin receptors. The other half lack the RI7 208 binding determinant, probably due to a mutation in one of their transferrin receptor gene copies [18, 34]. When mutant AKR1 cells bind multivalent IgM antibody, antibody-bearing receptors are internalized and assume an intracellular distribution indistinguishable from that of untreated receptors (J. Woods, unpublished immunolocalization studies), and cell-surface receptor expression is decreased [19]. Their escape from growth inhibition by RI7 208 antibody and their ability to internalize and down-regulate antibody-bound receptors appears to result from a reduction in crosslinking at the cell surface [19].

Though receptor crosslinking is often implicated in changes in surface expression and alterations in internal processing of receptors, it has not been studied systematically. Down-regulation of cell-surface receptors by exposure to anti-receptor antibodies has been observed in several systems, including receptors for insulin [38–41] epidermal growth factor [42], and antigen receptor on B cells (cell-surface Ig) [43]. Mellman *et al.* [44, 45] noted that down-regulation of macrophage

---

Fig. 10. Immunoperoxidase localization of RI7 217 IgG and RI7 208 IgM. AKR1 cells were fixed after 60 min incubation at 37°C in the presence of the antibody. (*A*), The RI7 217 antibody, presumably bound to transferrin receptors, is present in coated pits (cp) (inset), along the plasma membrane (pm), and intracellularly in endosomal structures, some of which have tubular extensions (arrows). (*B*), The RI7 208 antibody apparently causes the transferrin receptors to aggregate into patches along the plasma membrane (arrows). No RI7 208 antibody could be detected in any intracellular structures. In addition, the cytoplasm of cells incubated with RI7 208 appeared to much more sparse than in control cells or in cells incubated with RI7 217. There was much less endoplasmic reticulum (er) and the Golgi complex (go) was much less well organized. nu, nucleus, m, mitochondria. Bars, 200 μ*M*.

immunoglobulin Fc receptors upon interaction with polyvalent Ig containing complexes leads to increased receptor degradation in lysosomes. They suggested that crosslinking was important for this lysomal pathway because receptors with bound monovalent Fab anti-receptor antibody were recycled directly from endosomes, without entry into a lysomal compartment. Enns *et al.* [10] have suggested that crosslinking might be involved in induction of internalization of transferrin receptors.

By making fragments of anti-transferrin receptor antibodies we could study the effects of differences in antibody valence independently of possible differences between antibodies in binding site specificity. The two antibodies used here bind to different determinants of the transferrin receptor but can still cross-block each other's binding, indicating that the determinants are in close proximity [18, 34]. Cells exposed to antibodies and fragments were always grown in serum-containing medium, so that receptor levels were not influenced by changes in external iron or transferrin.

In this study, we found that culture in monovalent antibody fragments from either the IgM or the IgG antibody did not significantly affect cell growth (Fig. 3), surface receptor expression (Figs. 4 and 5), or receptor degradation (Fig. 6). Divalent antibody, either intact IgG_{2a} or divalent $F(ab')_2$ from IgM, caused a uniform down-regulation of cell-surface expression of all cells on short-term culture (Figs. 4 and 5), possibly by increasing the rate of internalization, but there were differences in other results between $F(ab')_2$ and intact IgG. The decrease in cell-surface receptors upon culture in $F(ab')_2$ was not accompanied by a detectable increase in receptor degradation rate (Fig. 6) or a retardation in cell growth (Fig. 3), which were consistently seen with intact IgG (RI7 217). This observation may suggest distinct mechanisms for increased receptor internalization (perhaps mediated by crosslinking) and increased receptor degradation (perhaps involving antibody Fc). Culture in IgM antibody completely inhibited cell growth (Fig. 3) and caused a redistribution of surface receptor that resulted in heterogenous levels of expression in the population with some cells appearing negative for receptor while most expressed receptor in varying amounts (Fig. 4 and Ref. [19]). The mechanism and significance of this heterogeneous expression are not understood (see also Ref. [19]).

Immunolocalization of receptor by anti-transferrin receptor antibody and fluorescein-labeled anti-Ig in fixed, steady-state AKR1 cells detected receptor dispersed on the cell surface and in a juxtanuclear, Golgi region in agreement with the transferrin receptor distribution seen in previous results using a myeloma cell line [13]. Culture in monovalent or divalent antibody fragments or intact IgG, followed by fixation and visualization with labeled anti-Ig, detected antibody in the same locations as receptor in steady-state cells. This suggests that the IgG antibody and antibody fragments followed the normal pathways of receptor distribution. Culture in IgM antibody showed a very different pattern of antibody distribution on immunolocalization. IgM antibody accumulated in patches and caps on the cell surface, with no antibody detectable intracellularly. This suggests that IgM antibody, probably due to extensive crosslinking on the cell surface,

prevents internalization of the receptor. The failure of AKR1 cells to internalize IgM-complexed receptor is consistent with our previous failure to detect an increase in receptor degradation in the presence of RI7 208 [19]. The appearance of about 20% of cells as antibody negative in RI7 208 is consistent with the flow cytometric measurements. It is not clear how these cells become negative, since no evidence of internalization of the antibody has been seen.

Immunolocalization did not detect a shift in intracellular distribution, which would be implied by increased degradation of receptors, in response to intact IgG. Weissman *et al.* [24] have pointed out that large changes in receptor degradation rate (receptor half-life) can be accomplished by quite small changes in recycling efficiency due to the rapid rate of receptor cycling (they estimate 12.5 min in K562 cells). Thus, the increase in receptor degradation seen upon culture in RI7 217 (IgG) may result from the shift of a relatively small proportion per cycle of the transferrin receptors to a degratory pathway without a visualizable change in total receptor distribution.

Mellman *et al.* [45] have shown that a monovalent Fab with specificity for macrophage Fc receptors is internalized into endosomes and recycled to the cell surface with little degradation. Our data suggest, also, that monovalent Fab anti-transferrin receptor follows the normal pathway of transferrin receptor cycling without perturbing that pathway. Divalent $F(ab')_2$, however, induces a down-regulation of surface transferrin receptor. Since no antibody Fc is present and since binding of monovalent Fab alone does not have this effect, down-regulation presumably results from the crosslinking of receptors. We could not detect any increase in receptor degradation upon culture in $F(ab')_2$, suggesting that the decrease in cell-surface expression may be a shift of receptors between cell surface and intracellular compartments. (Unfortunately, we have been unable to quantitate intracellular vs surface compartments because the lymphomas we have studied are lysed by treatments normally used to strip the cell surface (acid pH, proteolysis) [10–12, 23].) Another possible mechanism of reducing surface receptor without increased degradation is suggested by results of Rao *et al.* [46]. They observed about 50% reduction in surface transferrin receptors over 18 h in response to added iron. This reduction resulted from a decrease in receptor synthesis, detectable by 4 h, without any increase in degradation or change in receptor distribution between surface and intracellular locations. We have observed a similar down-regulation of surface receptors without an increase in receptor degradation in AKR1 cells upon addition of iron (unpublished results). Intact IgG antibody RI7 217, however, clearly does accelerate receptor degradation (Fig. 6 and Ref. [19]), presumably by diversion of some proportion of receptors each cycle to a lysosomal compartment [24, 44]. Weissman *et al.* [24] also observed a decrease in metabolically labeled [^{35}S]-methionine transferrin receptor half-life in K562 cells cultured in OKT9 (IgG anti-transferrin receptor). Further, they noted an increase in the rate of receptor synthesis, which modulated the loss of receptors.

Antibody-induced down-regulation of receptors reduces cell-surface receptor levels and would, therefore, be expected to reduce the cells' receptor-dependent

functions. Treatment of K562 cells with OKT9 (an IgG anti-human transferrin receptor) reduced iron uptake in the studies of Weissman *et al.* [24]. This mechanism may account for the moderate reduction in growth of AKR1 cells in the presence of intact RI7 217 seen in our studies. Damage to cells resulting from such a reduction in receptors will, of course, vary depending on particular cells' requirements for iron and on the extent of the down-shift. This probably accounts for the inhibition seen in some systems by IgG anti-transferrin receptor antibodies [16, 21, 25]. But the receptors remaining following down-regulation appear to function normally. This is suggested by our own results (growth continues, cloning efficiency is unaffected [19], and, in the present study, receptor localization is normal) and those of Weissman *et al.* [24] where OKT9 (IgG)-bound receptors continue to cycle and mediate iron uptake and of Taetle *et al.* [23] where B3/25 (IgG)-bound receptors continue to internalize transferrin.

AKR1 cells treated with IgM (RI7 208) anti-transferrin receptor antibody fail to take up iron [14, 18] and stop growing [14, 18, 19] (Fig. 3). Immunolocalization experiments presented here suggest that receptors with bound IgM are not internalized. Taetle *et al.* [23] also suggest that cells inhibited by the IgA anti-human transferrin receptor antibody 42/6 do not internalize receptor. However, why extreme crosslinking prevents internalization is unclear. Others have observed down-regulation of receptors by IgM antibodies [17, 22] but it is not known whether this down-regulation was the result of internalization of antibody.

The studies presented here demonstrate that effects of antibody binding on transferrin receptor distribution, metabolism, and function depend, at least in part, on antibody valence and therefore, inferentially, on the degree of crosslinking of receptors by antibody. We show two distinct mechanisms by which different degrees of antibody crosslinking can interfere with receptor function: by receptor down-regulation and by blocking internalization. Crosslinking alone can clearly influence the distribution of receptors between intracellular and cell-surface compartments. Crosslinking and other factors (antibody Fc?) may be involved in "sorting" receptors along different intracellular pathways (degradation, recycling, storage). Though these influences of antibody crosslinking cannot yet be related to the regulation of normal cycling pathways, they suggest mechanisms that might be involved in such regulation. Crosslinking by ligand has been proposed as a mechanism of activation of down-regulation for Fc receptors on macrophages [43] and for internalization of transferrin receptors [10, 47]. Further studies will be needed to understand how extensive crosslinking of receptors by multivalent complexes can inhibit internalization and thus block cell receptor function.

This work was supported by Grant CA-25893 from the National Cancer Institute. J. Woods is a Leukemia Society of America Special Fellow.

REFERENCES

1. Larrick, J. W., and Cresswell, J. (1979) *J. Supramol. Struct.* **11**, 579–586.
2. Barnes, D., and Sato, G. (1980) *Cell* **22**, 649–655.
3. Shindelman, J. E., Ortmeyer, A. E., and Sussman, H. H. (1981) *Int. J. Cancer* **27**, 329–334.

4. Sutherland, R., Delia, D., Schneider, C., Newman, R., Kemshead, J., and Greaves, M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4515–4519.
5. Trowbridge, I. S., and Omary, M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3039–3043.
6. Neckers, L. M., and Cossman, J. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3491–3498.
7. Karin, M., and Mintz, B. (1981) *J. Biol. Chem.* **256**, 3245–3252.
8. Bleil, J. D., and Bretscher, M. S. (1982) *EMBO J.* **1**, 351–355.
9. Ciechanover, A., Schwartz, A. L., and Lodish, H. F. (1983) *J. Cell. Biochem.* **23**, 107–130.
10. Enns, C. A., Larrick, J. W. Suomalainen, H., Schroder, J., and Sussman, H. H. (1983) *J. Cell Biol.* **97**, 579–585.
11. Hopkins, C. R., and Trowbridge, I. S. (1983) *J. Cell Biol.* **97**, 508–521.
12. Klausner, R. D., van Renswoude, J., Ashwell,G., Kempf, C., Schechter, A. N., Dean, A., and Bridges, K. R. (1983) *J. Biol. Chem.* **258**, 4715–4724.
13. Woods, J. W., Doriaux, M., and Farquhar, M. G. (1986) *J. Cell Biol.* **103**, 277–286.
14. Trowbridge, I. S., Lesley, J., and Schulte, R. (1982) *J. Cell Physiol.* **112**, 403–410.
15. Trowbridge, I. S., and Lopez, F. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1175–1179.
16. Taetle, R., Honeysett, J. M., and Trowbridge, I. S. (1983) *Int. J. Cancer* **32**, 343–349.
17. Larrick, J. W., and Hyman, E. S. (1984) *N. Engl. J. Med.* **311**, 214–218.
18. Lesley, J., and Schulte, R. (1984) *Mol. Cell Biol.* **4**, 1675–1681.
19. Lesley, J., and Schulte, R. (1985) *Mol. Cell Biol.* **5**, 1814–1821.
20. Ramensee, H. J., Lesley, J., Trowbridge, I., and Bevan, M.(1985) *Eur. J. Immunol.* **15**, 687–692.
21. Takai, F. (1985) *J. Immunol.* **134**, 1403–1407.
22. Vaickus, L., and Levy, R. (1985) *J. Immunol.* **135**, 1987–1997.
23. Taetle, R., Castagnola, J., and Mendelsohn, J. (1986) *Cancer Res.* **46**, 1759–1763.
24. Weissman, A. M., Klausner, R. D., Rao, K., and Harford, J. B. (1986) *J. Cell Biol.* **102**, 951–958.
25. Kemp, J. D., Thorson, J. A., McAlmont, T. H., Horowitz, M., Cowdery, J. S., and Ballas, Z. K. (1987) *J. Immunol.* **138**, 2422–2426.
26. Trowbridge, I. S. (1983) *in* Monoclonal Antibodies and Cancer, (Boss, B. D., Langman, R., Trowbridge, I., and Dulbecco, R., Eds.), pp. 53–61, Academic Press, New York.
27. Trowbridge, I. S., Newman, R. A., Domingo, D. L., and Sauvage, C. (1984) *Biochem. Pharmacol.* **33**, 925–932.
28. Trowbridge, I. S., and Newman, R. A. (1985) Monoclonal Antibodies To Transferrin Receptors, pp.235–262, Chapman & Hall, London.
29. Hyman, R., Cunningham, K., and Stallings, V. (1980) *Immunogenetics* **10**, 261–271.
30. Mage, M. G. (1980) *in* Methods in Enzymology (van Vunakis, H., and Langone, J. J., Eds.) Vol. 70, pp. 142–150, Academic Press, New York.
31. Omary, M. B., and Trowbridge, I. S. (1980) *J. Biol. Chem.* **255**, 1662–1669.
32. Lesley, J., Hyman, R., Schulte, R., and Trotter, J. (1984) *Cell. Immunol.* **83**, 14–25.
33. Weir, D. M. *in* Handbook of Experimental Immunology, Immunochimestry (Weir, D. M., Ed.) Vol.1, Blackwell Scientific, Oxford.
34. Lesley, J., and Schulte, R. (1986) *Immunogenetics* **24**, 163–170.
35. Trowbridge, I. S., and Hyman, R. (1975) *Cell* **6**, 279–287.
36. Mazia, D., Schatten, G., and Sale, W. (1975) *J. Cell Biol.* **66**, 198–200.
37. Platt, J., and Michael, A. F. (1983) *J. Histochem. Cytochem.* **31**, 840–842.
38. Granfeld, C. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2508–2511.
39. Roth, R. A., Maddux, B. A., and Cassell, D. J. (1983) *J. Biol. Chem.* **258**, 12,094–12,097.
40. Maron, R., Jackson, R. A., Jacobs, S., Eisenbarth, G., and Kahn, C. R. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7446–7450.
41. Taylor, S. I., and Marcus-Samuels, B. (1984) *J. Clin. Endocrinol. Metab.* **58**, 182–186.
42. Gregorion, M., and Rees, A. R. (1984) *EMBO J.* **3**, 929–937.
43. Taylor, R. B., Duffus, P. H., Raff, M. C., and dePetris, S. (1971) *Nature New Biol.* **233**, 225–229.
44. Mellman, I., and Putner, H. (1984) *J. Cell Biol.* **98**, 1170–1177.
45. Mellman, I., Putner,H., and Konan, P. U. K. (1984) *J. Cell Biol.* **98**, 1163–1169.
46. Rao, K. K., Shapiro, D., Mattia, E., Bridges, K., and Klausner, R. (1985) *Mol. Cell Biol.* **5**, 595–600.
47. Larrick, J. W., Enns, C., Raubitschek, A., and Weitraub, H. (1985) *J. Cell. Physiol.* **124**, 283–287.

Received August 2, 1988
Revised version received November 29, 1988

Printed in Sweden

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):.

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

VOL 73, NO 7 MAY 15, 1989

BLOOD

The Journal of
The American Society of
Hematology

PLEASE NOTE REVISED INFORMATION FOR CONTRIBUTORS

(See page xvi for details)

GRUNE & STRATTON
Harcourt Brace Jovanovich, Inc.

Inhibition of Colony-Stimulating Factor-1 Activity by Monoclonal Antibodies to the Human CSF-1 Receptor

By Charles J. Sherr, Richard A. Ashmun, James R. Downing, Masahiro Ohtsuka, Shirley G. Quan, David W. Golde, and Martine F. Roussel

Four of 12 monoclonal antibodies (MoAbs) directed to different epitopes in the extracellular domain of the human colony-stimulating factor-1 receptor (CSF-1R, the c-*fms* proto-oncogene product) specifically inhibit CSF-1 binding to receptor-bearing cells. All four antibodies abrogated CSF-1–dependent colony formation by human bone marrow-derived macrophage precursors and by mouse NIH-3T3 cells expressing a transduced human c-*fms* gene. In addition, one of these antibodies (designated MoAb 2-4A5) interfered with the ligand-independent proliferation of NIH-3T3 cells transformed by an oncogenic, mutant c-*fms* allele. Unlike CSF-1 itself, neither MoAb 2-4A5 nor the other three inhibitory antibodies (MoAbs 12-2D6, 12-3A1, and 12-3A3) induced CSF-1R internalization or degradation. These antibodies should prove useful not only for identifying and quantitating CSF-1R on receptor-bearing cells but for abrogating specific receptor signals that govern the proliferation and survival of human mononuclear phagocytes.

THE MACROPHAGE colony-stimulating factor (CSF-1 or M-CSF) is a lineage-restricted hematopoietic growth factor that stimulates the proliferation and supports the survival of cells of the mononuclear phagocyte series (reviewed in references 1 and 2). CSF-1 is a homodimeric glycoprotein that exerts its pleiotropic effects by binding to a single class of high affinity cell surface receptors expressed on monocytes, macrophages, and their committed bone marrow progenitors. The CSF-1 receptor (CSF-1R) is encoded by the c-*fms* proto-oncogene[3] and is one of a family of growth factor receptors that exhibits a ligand-activated tyrosine-specific protein kinase activity (reviewed in reference 4). The human receptor consists of a 512 amino acid extracellular domain that includes the CSF-1 binding site, a single 25 amino acid membrane-spanning segment, and a 435 amino acid cytoplasmic portion that includes the tyrosine kinase domain.[5,6] Activation of the receptor kinase in cells stimulated by CSF-1 triggers a cascade of biochemical responses that eventually culminate in DNA synthesis and mitogenesis.[2,7]

Introduction of retroviral vectors containing the cloned human c-*fms* cDNA into heterologous target cells results in CSF-1R expression and enables the cells to form colonies in semisolid medium in response to human recombinant CSF-1.[8] By inoculating v-*ras* transformed rat cells expressing transduced human CSF-1 receptors into newborn rats and fusing the spleens from tumor-bearing animals to rat myeloma cells, we obtained a series of monoclonal antibodies (MoAbs) reactive with different epitopes in the extracellular domain of the human CSF-1 receptor.[9,10] These MoAbs specifically immunoprecipitate metabolically labeled human (but not mouse or feline) CSF-1R molecules and support receptor tyrosine kinase activity in in vitro reactions performed with immune complexes. The antibodies have been successfully used to quantify CSF-1R expression on human peripheral blood monocytes, bone marrow mononuclear phagocyte precursors, and on malignant blast cells from a subset of patients with myeloid leukemia by use of fluorescence-activated flow cytometry.[10] The MoAbs are also useful for purifying human mononuclear phagocytes by antibody-dependent cell sorting techniques.

To determine whether any of these MoAbs have biological activity, either as direct activators of the CSF-1R kinase or as inhibitors of CSF-1 binding to its receptor, we screened 12 purified monoclonal immunoglobulin G (IgG) preparations for their ability to enhance or inhibit the CSF-1–dependent growth of mouse NIH-3T3 cells expressing human CSF-1R. None of the MoAbs acted as agonists in mimicking CSF-1 stimulation, but four antibodies inhibited the CSF-1 dependent growth of receptor-bearing cells. We show that these four MoAbs bind with high affinity to human CSF-1R, and at nanomolar concentrations, block CSF-1 binding to its receptor and specifically inhibit CSF-1 dependent colony formation. One of the MoAbs has the additional property of being able to inhibit the ligand-independent proliferation of cells transformed by an oncogenic, mutant human c-*fms* allele.

MATERIALS AND METHODS

MoAbs reactive to the human CSF-1 receptor. The preparation of rat MoAbs to human CSF-1R has been described in detail elsewhere.[10] In brief, transformed normal rat kidney (NRK) cells expressing a transduced human c-*fms* gene were inoculated into newborn Sprague-Dawley or Lewis rats, and spleens resected from animals mounting an immune response to human CSF-1R were fused to rat myeloma cells. Twelve individual hybridomas producing monoclonal IgG directed to human CSF-1R were cloned at limiting dilution in microtiter wells and then recloned from single colonies in semisolid medium. All hybridoma cell lines were adapted to serum-

From the Howard Hughes Medical Institute and Departments of Tumor Cell Biology, Hematology-Oncology, and Pathology, St Jude Children's Research Hospital, Memphis; and Division of Hematology-Oncology, UCLA School of Medicine, Los Angeles.

Submitted January 6, 1989; accepted January 16, 1989.

Supported by the Howard Hughes Medical Institute and US Public Health Service Grant No. CA47064 (C.J.S.); by Grants No. CA30388, CA32737, and HL42107 (D.W.G.); by Cancer Center CORE Grant No. CA21765 to St Jude Children's Research Hospital; and by the American Lebanese Syrian Associated Charities (S.J.C.R.H.).

Address reprint requests to Charles J. Sherr, MD, PhD, Howard Hughes Medical Institute, Department of Tumor Cell Biology, St Jude Children's Research Hospital, 332 N Lauderdale, Memphis, TN 38105.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" *in accordance with 18 U.S.C. section 1734 solely to indicate this fact.*

0006-4971/89/7307-0014$3.00/0

free medium, enabling purification of IgGs to homogeneity by conventional biochemical methods. MoAbs tested for growth inhibitory properties included the following 12 previously designated clones[10]: 2-4A5-4, 7-7A3-17, 9-2A1-2B5, 9-4D2-1E4, 14-1C1-2D10 (all γ1); 10-4B2-2E4, 12-2D6-2C7, 12-3A1-2B8, 12-3A3-1B10, 12-3D3-2F11 (all γ2a); 3-4A4-E4 (γ2b); and 12-6A4-2B6 (γ2c). For simplicity, the nomenclature for MoAbs in the text has been abbreviated to include only the first four or five characters (eg, 2-4A5, 12-2D6, etc).

*Colony-stimulating assays with c-*fms *transfected NIH-3T3 cell lines.* Procedures for transfection of mouse NIH-3T3 cells with vectors expressing cloned human c-*fms* and CSF-1 cDNAs, for deriving cell lines expressing these gene products, and for subcloning the cells in agar have been described in detail elsewhere.[8,11] All cloning experiments were performed using Iscove's modification of Dulbecco's medium containing 10% heat-inactivated fetal bovine serum (FBS), antibiotics, excess glutamine, and purified human recombinant CSF-1 where indicated. NIH-3T3 cells expressing the normal c-*fms* allele do not form colonies in semisolid medium in the absence of human CSF-1 but yield colonies in response to physiologic doses (10 pmol/L to 1 nmol/L) of the human recombinant growth factor.[8] Assays for colony formation with these cells routinely used a concentration of CSF-1 (5 nmol/L) in excess of that required for maximal colony forming efficiency. Under these conditions, approximately 30% of 10^4 cells plated per 60 mm culture dish form colonies containing more than 50 cells within 2 weeks of initiating the cultures.

Tumorigenic NIH-3T3 cells transformed by an "activated" human c-*fms* gene[11] or by the feline v-*fms* oncogene[12] form colonies in agar in the absence of CSF-1. We used NIH-3T3 cells transformed by a c-*fms* double mutant containing an activating mutation in its extracellular domain (substitution of serine for leucine 301) together with a complementing up-regulating mutation in its carboxylterminal tail (substitution of phenylalanine for tyrosine 969).[11] The feline v-*fms* oncogene carries analogous genetic alterations.[13] Both transformed cell lines form colonies at approximately 20% plating efficiency, and the number of colonies is increased approximately twofold by an excess of human CSF-1.[9,11] NIH-3T3 cells cotransfected with retroviral vectors containing both the human c-*fms* and human CSF-1 genes undergo transformation by an autocrine mechanism and also form colonies in agar in the absence of exogenous ligand.[8,14]

Colony-stimulating assays with human bone marrow cells. After appropriate informed consent was obtained, bone marrow from the iliac crest of healthy adult volunteers was aspirated into preservative-free heparin. Light density cells were purified by Ficoll-Hypaque gradient centrifugation (Pharmacia Laboratory, Piscataway, NJ),[10] and the mononuclear cell fraction was washed three times and suspended in Iscove's modified Dulbecco's medium (Irvine Scientific, Santa Ana, CA) containing 20% FBS (Irvine Scientific, screened lot). The cells were then allowed to adhere to plastic for two hours in a humidified incubator at 37°C with 8% CO_2 in air. The resultant light density nonadherent cells were plated in 24-well culture plates (Becton Dickinson, Lincoln Park, NJ) in Iscove's medium containing 20% FBS, 1 mmol/L L-glutamine, and 0.37% Bacto-Agar (Difco Laboratories, Detroit). Human recombinant CSF-1 was added directly to the wells, the agar cell suspension was mixed with the growth factor to give a final volume of 0.5 mL, and the mixture was allowed to cool and solidify in the well. Colonies defined as a cluster of more than 40 cells were counted using an inverted microscope 14 days later.

Competitive radioreceptor assay. Purified human recombinant CSF-1 produced by mammalian CHO cells (courtesy of Dr Steven Clark, Genetics Institute, Cambridge, MA) was radioiodinated to a specific activity of 1.2×10^8 cpm/μg^1 and tested for its ability to bind to NIH-3T3 cells expressing human CSF-1R. All steps were performed at 4°C to avoid ligand-induced internalization and degradation of receptors (downmodulation).[14,15] Cells seeded at 5×10^4 per well in 96-well microtiter plates were allowed to attach overnight, and were incubated in triplicate with increasing concentrations of ^{125}I-labeled CSF-1 in 0.1 mL of RPMI 1640 medium containing 10% heat-inactivated fetal calf serum and 10 mmol/L HEPES, pH 7.4, for 18 hours. After binding, the supernatants were collected, and the cells were washed three times with complete medium and lysed in phosphate-buffered normal saline containing 0.5% Nonidet P-40. Radioactivity in the supernatant and that bound to cells was determined in a gamma counter. Nonspecific binding determined in the presence of a 20-fold excess concentration of unlabeled human CSF-1 was <3% of the total bound radioactivity determined in the absence of competitor, even at ^{125}I-labeled CSF-1 concentrations as high as 500 pmol/L. These background levels were subtracted from the total bound cpm to calculate levels of specific binding. Apparent dissociation constants (K_D) were calculated by the method of Scatchard.[16] This approach may underestimate the actual binding affinity and generally renders values higher than those determined kinetically.[15] Competitive displacement assays were performed with the same preparation of radiolabeled ligand, using concentrations that occupied 50% of the total binding sites per well. Cells were preincubated for 60 minutes at 4°C with various concentrations of unlabeled human recombinant CSF-1 or with purified monoclonal IgGs before addition of the labeled growth factor. All subsequent steps were performed as described above.

Assays using identical methods to determine the K_Ds for binding of ^{125}I-labeled monoclonal IgGs to CSF-1R on the same cells were unsuccessful. MoAbs radioiodinated by the same protocol showed high levels of nonspecific binding, so that binding sites on receptor-positive NIH-3T3 cells could not be saturated using practical concentrations of ^{125}I-labeled antibodies. The apparent relative affinities of the MoAbs for receptor epitopes were therefore deduced from their ability to compete with radiolabeled ligand for binding sites, as compared with results obtained with unlabeled CSF-1.

Other analytical methods. Techniques for fluorescence-activated flow cytometry,[10] metabolic labeling with [^{35}S]methionine, preparation of cell lysates, immunoprecipitation, and electrophoresis of proteins in gels containing sodium dodecyl sulfate[17] were performed as described in the references cited.

RESULTS

Antibody-mediated inhibition of CSF-1 induced colony formation. To screen individual MoAbs for their ability to inhibit CSF-1–dependent cell proliferation, mouse NIH-3T3 cells expressing human CSF-1R were seeded in semisolid medium containing human recombinant CSF-1 (10,000 U/mL or ca. 5×10^{-9} mol/L) together with various concentrations of purified monoclonal IgGs. NIH-3T3 cells expressing the human c-*fms* gene are not morphologically transformed and do not form colonies in semisolid medium in the absence of human CSF-1.[8] Recombinant CSF-1, added at a concentration approximately fivefold in excess of that required to promote a maximal proliferative response in the assay, induced the formation of approximately 3,000 colonies per culture dish, representing approximately 30% of the total cells plated.

In the absence of CSF-1, none of the 12 monoclonal IgGs reactive to human CSF-1R stimulated colony formation at concentrations between 10^{-10} mol/L and 10^{-6} mol/L, indicating that none could act as receptor agonists. However,

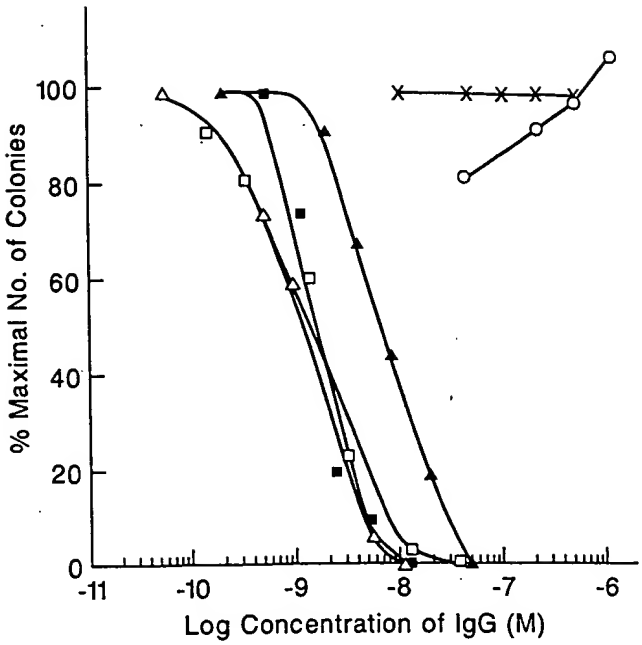


Fig 1. Antibody-mediated inhibition of colony formation by NIH-3T3 cells expressing human CSF-1R. Cells (10^4/dish) were seeded in 0.3% Noble agar in complete medium containing 10,000 U/mL human recombinant CSF-1 together with purified MoAbs at the indicated concentrations. Approximately 3,000 colonies per dish were obtained in the absence of antibodies. Symbols: MoAb 2-4A5 (▲); MoAb 12-2D6 (△); MoAb 12-3A3 (■); MoAb 12-3A1 (□); MoAb 7-7A3 (○); and MoAb 10-4B2 (x).

four of the MoAbs (2-4A5, 12-2D6, 12-3A1, and 12-3A3) inhibited CSF-1–dependent colony formation (Fig 1). The concentration yielding 50% inhibition (ID_{50}) of colony formation for three of the antibodies (12-2D6, 12-3A1, and 12-3A3) was 2×10^{-9} mol/L, whereas MoAb 2-4A5 was somewhat less effective and had an ID_{50} of 8×10^{-9} mol/L. The remaining eight antireceptor MoAbs showed no inhibitory activity; representative results with two of the latter antibodies (an isotype-matched γ2a and γ1) are also shown in Fig 1.

Although this experimental system is as sensitive as any CSF-1 bioassay,[2] mesenchymal cell lines such as mouse NIH-3T3 cells do not normally express endogenous CSF-1R nor respond to the growth factor. To generalize the results to a physiologically relevant system, we therefore tested the ability of these MoAbs to inhibit macrophage colony formation from human bone marrow progenitor cells. When nonadherent human bone marrow cells were plated at densities between 2 and 4×10^5 cells/mL in semisolid medium in the presence of increasing concentrations of human recombinant CSF-1, the maximal response was obtained with 1 nmol/L of the growth factor (Fig 2A). At 14 days after plating, the colonies contained large, dispersed cells consisting of pure nonspecific esterase-positive macrophages. In repeated experiments, some stimulation of macrophage colony formation was routinely observed at CSF-1 concentration as low as 50 pmol/L. Paradoxically, human CSF-1 has been reported to be significantly more active on mouse than on human bone marrow progenitors, stimulating fewer macrophage colonies from human bone marrow than that expected from titration of murine cells.[18-21] The frequency of macrophage colonies obtained using human bone marrow and the higher CSF-1 dose response range (50 pmol/L to 1 nmol/L) as compared with results with mouse bone marrow progenitors (1 to 500 pmol/L)[1,2] were in agreement with these earlier reports.

In the presence of 100 pmol/L CSF-1, MoAbs 2-4A5, 12-2D6, 12-3A1, and 12-3A3 completely inhibited macrophage colony formation at the lowest concentration of antibody tested (6.5×10^{-9} mol/L) (Fig 2B). In contrast, three other antireceptor MoAbs (9-4D2, 10-4B2, and 14-1C1) had no inhibitory or agonistic effects, even at threefold higher antibody concentration. Thus, antireceptor MoAbs that inhibited CSF-1–dependent proliferation of NIH-3T3 cells expressing human CSF-1R also abrogated CSF-1–dependent macrophage colony formation by human bone marrow progenitor cells, whereas other antireceptor MoAbs had no activity in either experimental system.

Inhibition of autocrine transformation by CSF-1. NIH-3T3 cells transformed by cotransfection with human CSF-1 and c-*fms* genes form colonies in agar in the absence of exogenous CSF-1.[8] Neutralizing antibodies to the growth factor can abrogate colony formation by these autocrine

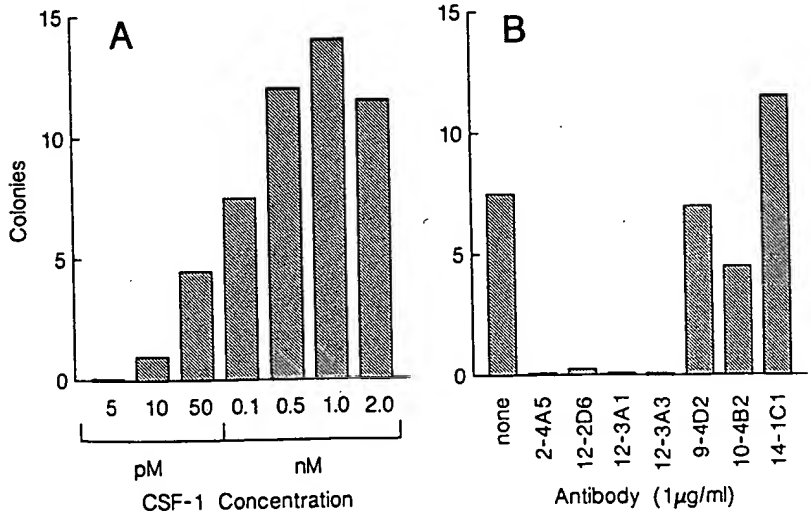


Fig 2. Antibody-mediated inhibition of colony formation by human bone marrow-derived macrophages. (A) Dose response to CSF-1. The number of colonies per 2×10^5 cells plated is shown at growth factor concentrations between 5 pmol/L and 2 nmol/L. (B) Inhibition of macrophage colony formation. Cultures of human bone marrow cells contained 2×10^5 cells/mL, 100 pmol/L recombinant CSF-1 and the MoAbs indicated. Bar graphs in *A* and *B* indicate averages from triplicate determinations in one of three representative experiments.

Table 1. Antibody-Mediated Inhibition of Colony Formation by Transformed Mouse NIH-3T3 Cells Expressing Both Human CSF-1 and CSF-1R

Antibody Added	Efficiency of Colony Formation (% of Cells Plated)
None	24.4
2-4A5	0.4
12-2D6	1.6
12-3A1	1.2
12-3A3	0.8
7-7A3	22.4

Cells were plated in 0.3% Noble agar in the absence of CSF-1. Purified monoclonal IgGs were added to the cultures at concentrations of 20 μg/mL (ca. 1.3×10^{-7} mol/L). Colonies containing more than 40 cells were enumerated nine days after plating.

transformants, but the proliferation of subclones producing relatively high concentrations of CSF-1 tends to be at least partially refractory to such inhibition.[14] Autocrine transformants were also inhibited from forming colonies by antireceptor MoAbs 2-4A5, 12-2D6, 12-3A1, and 12-3A3. As shown in Table 1, colony formation was arrested by >95% at antibody concentrations of 1.3×10^{-7} mol/L, whereas a noninhibitory control antibody to CSF-1R (MoAb 7-7A3) had no effect on colony growth. The concentrations of monoclonal IgGs necessary to inhibit the frequency of colony formation of autocrine transformants by 95% were five- to tenfold higher than those required to inhibit colony formation of receptor-bearing cells in response to high concentrations (5 nmol/L) of exogenously added CSF-1 (Fig 1). This shows that even though the probability of effective ligand-receptor interactions is maximized in cells that simultaneously express the growth factor and its receptor, signal transduction can still be interrupted by exogenous antireceptor antibodies.

MoAb 2-4A5 inhibits the growth of cells transformed by a mutant c-fms *allele.* Critical structural mutations within the c-*fms* protooncogene can unmask its oncogenic potential by constitutively activating the CSF-1R kinase, thereby enabling it to transform cells in the absence of ligand.[8,11,13] Substitution of serine for leucine at position 301 within the extracellular domain of human CSF-1R is sufficient to activate c-*fms* transforming activity in mouse NIH-3T3 cells.[11] Unlike cells expressing the normal CSF-1 receptor, NIH-3T3 cells expressing activated mutant CSF-1 receptors form colonies in agar in the absence of CSF-1. Although the activating mutation induces a conformational change that increases the receptor kinase activity in the absence of CSF-1, the mutant receptor retains a high affinity ligand-binding site and can be further up-regulated by the growth factor. Additional genetic alterations at the receptor carboxylterminus (eg, substitution of phenylalanine for tyrosine 969) can also up-regulate the receptor kinase activity, but the latter changes are themselves insufficient to confer transforming activity.[8]

To determine whether MoAbs directed against CSF-1R might inhibit the growth of transformed cells expressing activated receptors, NIH-3T3 cells transformed by a mutant c-*fms* allele were seeded in semisolid medium together with individual MoAbs that were shown above to inhibit CSF-1–induced proliferation (Table 2). As controls, cells expressing the wild-type c-*fms* gene were unable to form colonies in agar in the absence of human recombinant CSF-1, but formed colonies at high plating efficiency when CSF-1 was added. Addition of MoAbs 12-2D6, 12-3A1, 12-3A3, and 2-4A5 at a concentration of 3.2×10^{-8} mol/L completely inhibited CSF-1–dependent colony formation. When transformed cells expressing the feline v-*fms* oncogene were plated in parallel, they formed colonies in both the absence or presence of CSF-1. The colony forming efficiency of v-*fms* transformants was enhanced 1.5- to twofold by the presence of the human recombinant growth factor that binds to the feline gene product with high affinity and further up-regulates its kinase activity.[9,11] As expected, no effect of the MoAbs on the latter cells was observed (Table 2), because the antibodies are species-restricted and do not react with the feline v-*fms* gene product.[9,10] Thus, the MoAb IgG preparations were not generally cytotoxic to transformed cells but specifically inhibited the CSF-1–dependent growth of cells expressing human CSF-1R.

When cells transformed by a human c-*fms* gene containing both an activating (ser for leu 301) and a regulatory (phe for tyr 969) mutation were plated in agar, they also formed colonies in the absence of CSF-1 and their cloning efficiency was enhanced twofold by ligand addition. MoAbs 12-2D6, 12-3A1, and 12-3A3 did not inhibit the growth of these transformed cells in the absence of ligand, but reduced their plating efficiency in the presence of CSF-1 to the levels observed in medium lacking CSF-1 (Table 2). The latter effect was anticipated, based on the ability of the MoAbs to inhibit that component of the response that is CSF-1 dependent. In contrast, MoAb 2-4A5 significantly inhibited the

Table 2. Antibody-Mediated Inhibition of Colony Formation by Mouse NIH-3T3 Cells Expressing Normal or "Activated" Human CSF-1 Receptors

Cell Line Expressing	Efficiency of Colony Formation (% of Cells Plated)									
	−CSF-1					+CSF-1				
	−MoAb	+12-2D6	+12-3A1	+12-3A3	+2-4A5	−MoAb	+12-2D6	+12-3A1	+12-3A3	+2-4A5
c-*fms*	<0.01	<0.01	<0.01	<0.01	<0.01	31	0.9	0.4	0.5	0.2
v-*fms*	22	24	21	NT	27	42	34	37	NT	36
c-*fms* [S301, F969]	18	25	23	25	1.4	37	24	20	23	2.6

Cells were plated in 0.3% Noble agar in the absence (−CSF-1) or presence (+CSF-1) of 10,000 U/mL (ca. 5×10^{-9} mol/L) human recombinant CSF-1. Purified MoAbs were added to the cultures at concentrations of 5 μg/mL (ca. 3.2×10^{-8} mol/L). Colonies containing >50 cells were enumerated 14 to 18 days after plating.

Abbreviation: NT, not tested.

growth of these transformed cells either in the presence or absence of CSF-1, reducing their colony forming efficiency to <3% of cells plated. From these data, we conclude that the mechanism of inhibition of cell growth mediated by MoAb 2-4A5 differs from that of the other three inhibitory antibodies and does not depend on CSF-1.

The four inhibitory MoAbs compete with CSF-1 for receptor binding. To determine whether the growth inhibitory MoAbs interfere with ligand binding to its receptor, competitive radioreceptor assays were performed using ^{125}I-labeled human recombinant CSF-1. All assays were performed at 4°C to prevent ligand-induced receptor internalization and degradation. Under these conditions, the apparent K_D determined by Scatchard analysis[16] for binding of ^{125}I-labeled human recombinant CSF-1 was 9.4×10^{-11} mol/L, in good agreement with our previously obtained values.[11]

Figure 3 shows that in a competitive radioreceptor assay performed with the same labeled ligand preparation, unlabeled CSF-1 initially competed for binding sites on receptor-bearing NIH-3T3 cells at concentrations in the 10^{-11} mol/L range and displaced all of the labeled ligand at a concentration of ca. 10^{-9} mol/L. When prebound to the cells for 30 minutes before addition of radiolabeled CSF-1, MoAbs 12-3A1 and 12-2D6 were only about twofold less effective on a molar basis in competing for receptor binding of radiolabeled CSF-1 to the cells. MoAb 2-4A5 was tenfold less effective, and MoAb 12-3A3 was intermediate in inhibitory activity. The relative efficiency of these MoAbs in competing with CSF-1 for receptor binding sites on these cells was consistent with their hierarchy of inhibitory activity for ligand dependent colony formation (compare Figs 1 and 3). However, their ID_{50}s in the competitive radioreceptor assay were lower than those required for inhibition of colony formation, principally because the binding assay was performed for only 18 hours using limiting concentrations of ^{125}I-labeled CSF-1. Figure 3 also shows that an antireceptor MoAb that binds to epitopes in the CSF-1R extracellular domain but that lacks growth inhibitory activity did not interfere with ^{125}I-labeled CSF-1 binding to its receptor.

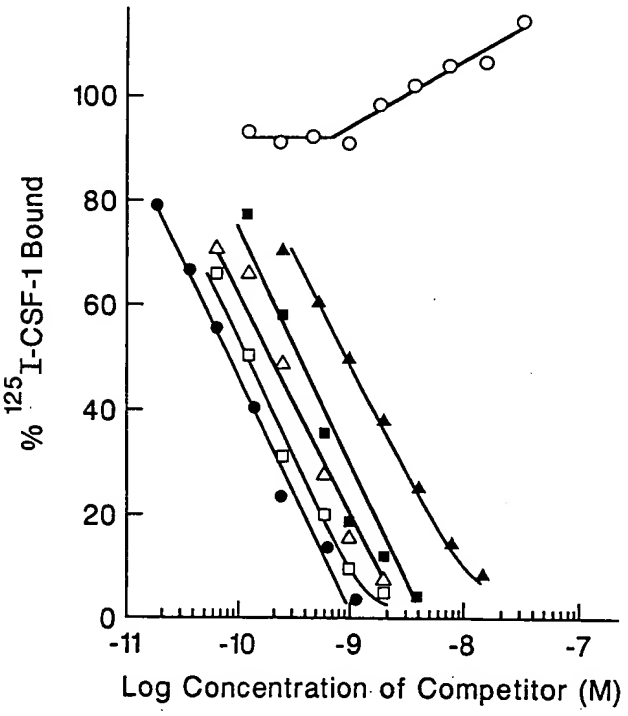


Fig 3. Competitive radioreceptor assay. Cells (5×10^4/well) were preincubated for 30 minutes with CSF-1 (●) or with MoAb IgGs before addition of ^{125}I-labeled CSF-1 for 18 hours at 4°C. The MoAbs included: 2-4A5 (▲); 12-2D6 (△); 12-3A3 (■); 12-3A1 (□); and 7-7A3 (○). The percentage of ^{125}I-labeled CSF-1 bound in the absence of competitor (ca. 32,000 cpm/well) was normalized to 100% (ordinate).

Although the ability of certain MoAbs to compete for CSF-1 binding might imply that they recognize epitopes within the ligand-binding pocket of CSF-1R, reciprocal binding data argue against this possibility. When a saturating concentration of unlabeled CSF-1 was bound to receptor-positive NIH-3T3 cells at 4°C, the subsequent binding of the antireceptor MoAbs was not detectably inhibited. As a representative example, Fig 4A shows that MoAb 2-4A5 generated a positive fluorescence profile when added to receptor-bearing cells that had prebound a saturating amount of ligand. To verify that the amounts of prebound CSF-1 were indeed sufficient to occupy all receptor binding sites before MoAb addition, an aliquot of the CSF-1–treated cells was incubated at 37°C for 60 minutes before addition of MoAb 2-4A5, and the cells were reanalyzed for expression of receptor epitopes by flow cytometry. After the temperature shift to 37°C, CSF-1R was completely downregulated in response to prebound ligand, resulting in the loss of all detectable receptor epitopes at the cell surface (Fig 4A). Thus, the concentration of CSF-1 prebound at 4°C, although sufficient to occupy all available binding sites and thereby induce complete receptor internalization at 37°C, did not interfere with MoAb binding at 4°C. Similar data were obtained with MoAbs 12-2D6, 12-3A1, and 12-3A3 (not shown), indicating that none of these reagents react with receptor epitopes that become masked by bound ligand.

The growth inhibitory MoAbs do not induce receptor downmodulation. The above results showed that the four growth inhibitory MoAbs directly interfere with CSF-1 binding to its receptor, but did not explain the differential effect of MoAb 2-4A5 in inhibiting the growth of cells transformed by activated c-*fms* mutants. Because physiological substrates for the CSF-1R receptor kinase may reside at the cell surface, one possibility is that MoAb 2-4A5 induces receptor internalization and degradation, thereby reducing the ligand-independent kinase activity of mutant CSF-1R molecules on the plasma membrane. Antibodies to the transforming *neu* (*erb*B-2, HER-2) gene product appear to inhibit cell growth by such a mechanism.[22] To test this possibility, NIH-3T3 cells expressing CSF-1R were stained either at 4°C or at 37°C with MoAb 2-4A5 and analyzed for antibody-dependent fluorescence by flow cytometry. Figure 4B shows that there was no significant diminution in the fluorescence profile after antibody treatment for two hours at 37°C, indicating that MoAb 2-4A5 did not induce inter-

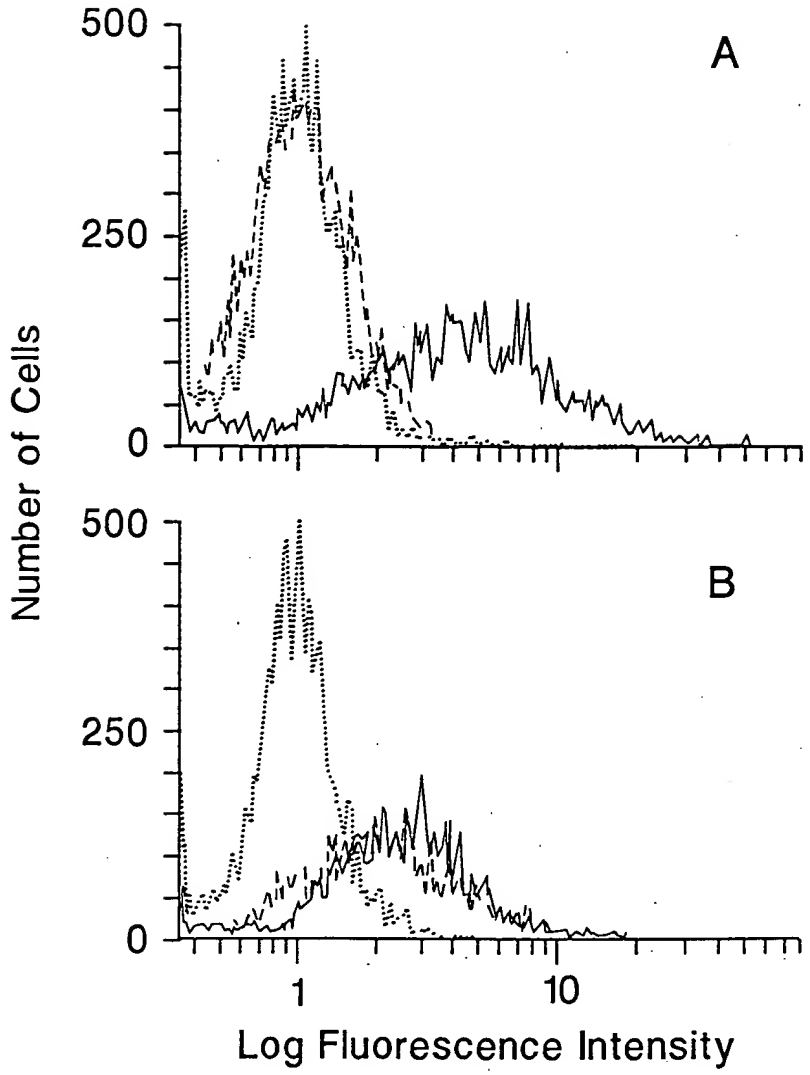


Fig 4. Flow cytometric assays for MoAb binding and CSF-1R downmodulation. (A) CSF-1R–positive NIH-3T3 cells were incubated with a saturating concentration of human CSF-1 for 1.5 hours at 4°C and then stained at 4°C with antireceptor MoAb 2-4A5 (——) or with an isotype-matched irrelevant MoAb (· · · ·) before counterstaining with goat anti-rat IgG. An aliquot of CSF-1–treated cells was shifted to 37°C for one hour before staining with MoAb 2-4A5 and goat anti-rat IgG at 4°C (– – –) in order to demonstrate that the prebound CSF-1 could downmodulate the receptor. (B) CSF-1R–positive NIH-3T3 cells were stained for two hours at 4°C (——) or at 37°C (– – –) with MoAb 2-4A5 before counterstaining with goat anti-rat IgG at 4°C. Control staining was performed at 4°C with an irrelevant isotype-matched antibody (· · · · ·).

nalization of CSF-1R. This assay is sufficiently sensitive to detect complete ligand-induced downmodulation of CSF-1R within 90 minutes at 37°C (Fig 4A). Similar negative results were obtained with MoAbs 12-2D6, 12-3A1, and 12-3A3.

To confirm these findings, the cells were metabolically labeled for 15 minutes with [^{35}S]methionine and then incubated for various times in medium containing excess unlabeled methionine either in the presence or absence of CSF-1 or different MoAbs. The cells were then lysed with detergent, and radiolabeled CSF-1R molecules were precipitated with antiserum and separated on denaturing polyacrylamide gels (Fig 5). In the absence of MoAb or CSF-1 treatment (top panel), the immature intracellular form of CSF-1R (designated gp130) was rapidly converted to the mature cell surface form of the receptor (gp150) which then turned over with a half-life of approximately three hours.[14] Addition of MoAb 2-4A5 to the cells during the "chase" period did not accelerate receptor degradation (Fig 5), whereas the addition of saturating concentrations of CSF-1 led to greatly increased receptor turnover.[14,15,23,24] Identical results to that obtained with MoAb 2-4A5 were also obtained with MoAbs 12-2D6, 12-3A1, and 12-3A3. Thus, binding of these antibodies to the receptor at 37°C leads neither to receptor internalization (Fig 4B) nor to accelerated turnover (Fig 5). MoAb 2-4A5 must therefore inhibit the growth of transformed cells by another mechanism.

DISCUSSION

Four of 12 MoAbs directed to epitopes in the extracellular domain of the human CSF-1 receptor were found to inhibit ligand-dependent colony formation by human macrophage precursors and by NIH-3T3 cells expressing a transduced human c-*fms* gene. In the latter system, the formation of colonies in response to exogenously added CSF-1 or resulting from autocrine stimulation were both suppressed by these MoAbs. Under conditions of reduced temperature at which receptor downmodulation does not occur, pretreatment of receptor-bearing cells with each of the four growth-inhibitory antibodies blocked subsequent CSF-1 binding, whereas prebinding of CSF-1 did not reciprocally inhibit the reactivity of the MoAbs to CSF-1R. These findings suggest that the

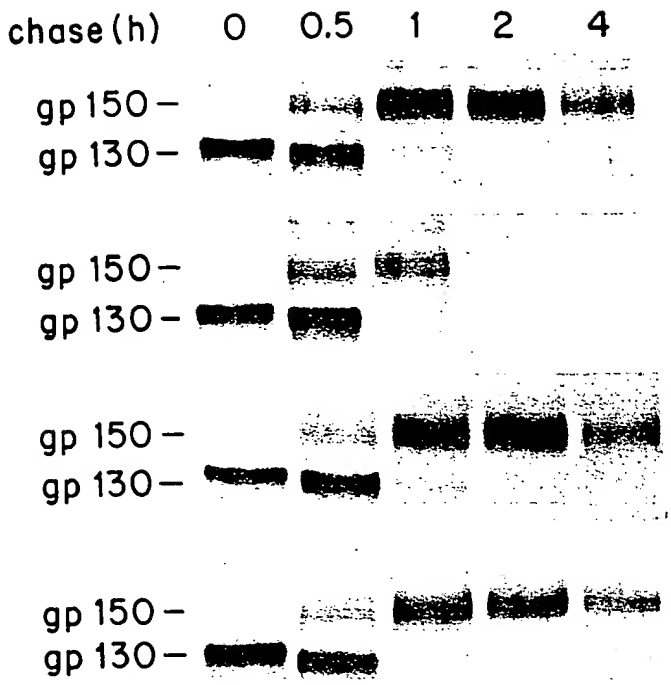


Fig 5. Receptor turnover in the presence or absence of MoAbs. CSF-1R–positive NIH-3T3 cells labeled for 15 minutes with [^{35}S]methionine were chased in the presence of unlabeled methionine for the indicated times in hours. Human recombinant CSF-1 (5 nmol/L) or MoAb 2-4A5 (20 μg/mL) were added during the chase period as indicated at the right margin. The positions of the immature (gp130) and mature (gp150) CSF-1R glycoproteins are indicated at the left margin.

MoAbs do not react with epitopes within the CSF-1 binding pocket but either sterically inhibit ligand binding or alter the receptor conformation to decrease its affinity for CSF-1. Because radioiodination of these MoAbs led to their partial denaturation, we were unable to directly measure the K_Ds for antibody-receptor interactions by equilibrium binding. However, the antibodies competed with ^{125}I-labeled ligand for receptor binding sites at subnanomolar concentrations that approximated those at which CSF-1 itself was an effective competitor. Therefore, the MoAbs must bind to CSF-1R on live cells with high affinity.

In colony inhibition assays performed with high concentrations of exogenously added CSF-1, MoAbs 12-2D6, 12-3A1, and 12-3A3 completely inhibited ligand-stimulated cell growth at concentrations of about 1 μg/mL, whereas MoAb 2-4A5 was about fivefold less potent. Nevertheless, MoAb 2-4A5 had the additional property of inhibiting the CSF-1 independent growth of cells transformed by an activated c-*fms* allele. Inhibition of colony formation was specifically mediated through a direct effect of MoAb 2-4A5 on human CSF-1R, since cells transformed by the feline v-*fms* gene product, which is not recognized by the antibody, were not growth arrested. Flow cytometry performed with cells expressing chimeric receptor molecules composed of different portions of the human CSF-1R extracellular domain fused to the remainder of the feline v-*fms* product[9] localized the epitopes for MoAbs 12-2D6, 12-3A1, and 12-3A3 between CSF-1R residues 1 to 308. In contrast, MoAb 2-4A5 binds to an epitope located between residue 349 and the transmembrane segment at amino acids 512-537 (R.A.A., M.F.R., and C.J.S., unpublished data). By use of both flow cytometric assays and kinetic receptor turnover studies, we found that none of these antibodies induced receptor internalization or degradation, indicating that the inhibitory effect of MoAb 2-4A5 on c-*fms* transformed cells was not due to induction of CSF-1R downmodulation. Taken together, these results imply that MoAb 2-4A5 acts directly on CSF-1R molecules at the cell surface to extinguish receptor-mediated signals for cell growth.

MoAb 2-4A5 might potentially interfere with conformational changes in CSF-1R that are required to activate the receptor kinase or ensure its interaction with relevant substrates. Under normal circumstances, such changes are induced by CSF-1 binding, but they can also be caused by "activating mutations" that up-regulate the receptor kinase in the absence of ligand.[11,13] It remains unclear whether the conformational changes that accompany kinase activation are intramolecular, or whether intermolecular events, such as the formation of receptor dimers or interaction with heterologous membrane proteins, are required for signal transduction across the membrane. Precedents for the latter possibility come from studies of other receptor systems.[25-28] Antibody-mediated reversal of an enzymatically active CSF-1R conformation might therefore reflect the ability of certain MoAbs to interfere with receptor aggregation, as opposed to direct effects on the folding of the monomeric glycoprotein molecules.

The fact that the human c-*fms* gene product can be activated as an oncogene by critical genetic alterations raises the possibility that similar genetic events occurring at the c-*fms* locus in situ might contribute to the abnormal proliferation of CSF-1R–bearing cells of the mononuclear phagocyte lineage. Indeed, surveys for the presence of such activating mutations in cases of myeloid leukemia are just beginning. If activated receptors could be demonstrated to contribute etiologically to a subset of such malignancies, MoAbs like 2-4A5 might ultimately prove to be of therapeutic value in arresting the growth of such tumor cells.

Similarly, MoAbs 2-4A5, 12-2D6, 12-3A1, and 12-3A3 might inhibit the proliferation of that subset of myeloid leukemias that coexpress CSF-1 and its receptor.[29] Because CSF-1 supports both the proliferation and survival of mononuclear phagocytes, MoAbs that inhibit responses to CSF-1 may also affect the survival of monocytes and macrophages and thereby be useful in the management of inflammatory disorders or of diseases involving latent microbial infection of phagocytic cells.

ACKNOWLEDGMENT

We thank Joy Browne, Virgil Holder, Carol Bockhold, Cheryl Trigg, and Edward Wingfield for expert technical assistance and Dr Steven Clark of Genetics Institute, Cambridge, MA, for recombinant human CSF-1.

REFERENCES

1. Stanley ER: The macrophage colony stimulating factor, CSF-1, in Colowick SP, Kaplan NO (eds): Methods in Enzymology-Immunochemical Techniques. San Diego, Academic, 1985, 116

2. Sherr CJ, Stanley ER: Colony stimulating factor-1, in Sporn MB, Roberts AB (eds): Peptide Growth Factors and Their Receptors. Heidelberg, Springer-Verlag, (in press)

3. Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER: The c-*fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell 41:665, 1985

4. Hanks SK, Quinn AM, Hunter T: The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. Science 241:42, 1988

5. Coussens L, Van Beveren C, Smith D, Chen E, Mitchell RL, Isacke CM, Verma IM, Ullrich A: Structural alteration of viral homologue of receptor proto-oncogene *fms* at carboxyl terminus. Nature 320:277, 1986

6. Sherr CJ: The *fms* oncogene. BBA Reviews on Cancer, Amsterdam, Elsevier, 948:225, 1988

7. Sherr CJ, Rettenmier CW, Roussel MF: Macrophage colony stimulating factor, CSF-1, and its proto-oncogene encoded receptor. Cold Spring Harbor, NY, Symp Quant Biol (in press)

8. Roussel MF, Dull TJ, Rettenmier CW, Ralph P, Ullrich A, Sherr CJ: Transforming potential of the c-*fms* proto-oncogene (CSF-1 receptor). Nature 325:549, 1987

9. Roussel MF, Downing JR, Ashmun RA, Rettenmier CW, Sherr CJ: CSF-1 mediated regulation of a chimeric c-*fms*/v-*fms* receptor containing the v-*fms*–coded tyrosine kinase domain. Proc Natl Acad Sci USA 85:5903, 1988

10. Ashmun RA, Look AT, Roberts WM, Roussel MF, Seremetics ST, Ohtsuka M, Sherr CJ: Monoclonal antibodies to the human CSF-1 receptor (c-*fms* proto-oncogene product) detect epitopes on normal mononuclear phagocytes and on human myeloid leukemic blast cells. Blood 72:827, 1989

11. Roussel MF, Downing JR, Rettenmier CW, Sherr CJ: A point mutation in the extracellular domain of the human CSF-1 receptor (c-*fms* proto-oncogene product) activates its transforming potential. Cell 55:979, 1988

12. Donner L, Fedele LA, Garon CF, Anderson SJ, Sherr CJ: McDonough feline sarcoma virus: Characterization of the molecularly cloned provirus and its feline oncogene (v-*fms*). J Virol 41:489, 1982

13. Woolford J, McAuliffe A, Rohrschneider LR: Activation of the feline c-*fms* proto-oncogene: Multiple alterations are required to generate a fully transformed phenotype. Cell 55:965, 1988

14. Rettenmier CW, Roussel MF, Ashmun RA, Ralph P, Price K, Sherr CJ: Synthesis of membrane-bound colony-stimulating factor-1 (CSF-1) and downmodulation of CSF-1 receptors in NIH 3T3 cells transformed by cotransfection of the human CSF-1 and c-*fms* (CSF-1 receptor) genes. Mol Cell Biol 7:2378, 1987

15. Guilbert LJ, Stanley ER: The interaction of ^{125}I–colony-stimulating factor-1 with bone marrow-derived macrophages. J Biol Chem 261:4024, 1986

16. Scatchard G: The attractions of proteins for small molecules and ions. Ann NY Acad Sci 51:660, 1949

17. Anderson SJ, Gonda MA, Rettenmier CW, Sherr CJ: Subcellular localization of glycoproteins encoded by the viral oncogene v-*fms*. J Virol 51:730, 1984

18. Waheed A, Shadduck RK: Purification of colony stimulating factor by affinity chromatography. Blood 60:238, 1982

19. Motoyoshi K, Suda T, Kusumoto K, Takaku F, Miura Y: Granulocyte-macrophage colony stimulating and binding activities of purified human urinary colony stimulating factor to murine and human bone marrow cells. Blood 60:1378, 1982

20. Das SK, Stanley ER: Structure-function studies of a colony stimulating factor (CSF-1). J Biol Chem 257:13679, 1982

21. Ralph P, Warren MK, Lee MT, Csejtey J, Weaver JF, Broxmeyer HE, Williams DE, Stanley ER, Kawasaki ES: Inducible production of human macrophage growth factor, CSF-1. Blood 68:633, 1986

22. Drebin JA, Link VC, Stern DF, Weinberg RA, Greene MA: Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. Cell 41:695, 1985

23. Wheeler EF, Rettenmier CW, Look AT, Sherr CJ: The v-*fms* oncogene induces factor independence and tumorigenicity in CSF-1 dependent macrophage cell line. Nature 324:377, 1986

24. Downing JR, Rettenmier CW, Sherr CJ: Ligand-induced tyrosine kinase activity of the colony stimulating factor-1 receptor in a murine macrophage cell line. Mol Cell Biol 8:1795, 1988

25. Yarden Y, Schlessinger J: Self-phosphorylation of epidermal growth factor receptor: Evidence for a model of intermolecular allosteric activation. Biochemistry 26:1434, 1987

26. Yarden Y, Schlessinger J: Epidermal growth factor induces rapid, reversible aggregation of the purified epidermal growth factor receptor. Biochemistry 26:1443, 1987

27. Böni-Schnetzler M, Pilch PF: Mechanism of epidermal growth factor receptor autophosphorylation and high affinity binding. Proc Natl Acad Sci USA 84:7832, 1987

28. Böni-Schnetzler M, Kaligian A, Del Vecchio R, Pilch PF: Ligand-dependent intersubunit association within the insulin receptor complex activates its intrinsic kinase activity. J Biol Chem 263:6822, 1988

29. Rambaldi A, Wakamiya N, Vellenga E, Horiguchi J, Warren MK, Kufe D, Griffin JD: Expression of the macrophage colony stimulating factor and c-*fms* genes in human acute myeloblastic leukemia cells. J Clin Invest 81:1030, 1988

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

September 1, 1989

Cancer Research

OFFICIAL JOURNAL OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH

VOLUME 49 • NO. 17 • PP 4677–4954
ISSN 0008-5472 • CNREA 8

[CANCER RESEARCH 49, 4906–4912, September 1, 1989]

Endocytosis and Degradation of Monoclonal Antibodies Targeting Human B-Cell Malignancies[1]

Oliver W. Press,[2] Andrew G. Farr, K. Ingrid Borroz, Susan K. Anderson, and Paul J. Martin

Departments of Medicine [O. W. P., K. I. B., S. K. A., P. J. M.] and Biological Structure [O. W. P., A. G. F., S. K. A.], University of Washington, and the Fred Hutchinson Cancer Research Center [O. W. P., P. J. M.], Seattle, Washington

ABSTRACT

Seven murine monoclonal antibodies (MoAbs) recognizing differentiation antigens present on B-lymphocytes were analyzed in preclinical studies for their potential use for antibody-targeted therapy of B-cell malignancies. MoAbs HD37 (anti-CD19), 1F5 (anti-CD20), HD6 (anti-CD22), MB-1 (anti-CD37), G28-5 (anti-CDw40), 7.2 (anti-class II), and DA4-4 (anti-IgM) were studied for their binding avidities, immunoreactivities, isotypes, endocytosis rates, degradation rates, and number of binding sites on Daudi cells. Lineweaver-Burke analyses of ^{125}I-labeled MoAbs demonstrated immunoreactivities ranging from 59 to 92%. Scatchard analyses of ^{125}I-MoAbs demonstrated that five of the antibodies had binding avidities in excess of 10^9 L/M, whereas MoAbs 1F5 and HD37 had avidities of 3–4 × 10^8 L/M. CD20, CD37, μ, and HLA Class II were found to be highly expressed (200,000–400,000 binding sites/cell) on Daudi cells whereas CD19, CD22, and CDw40 were less densely expressed (80,000–100,000 sites/cell). DA4-4 (μ), HD6 (CD22), and G28-5 (CDw40) were rapidly internalized by cells, HD37 (CD19) and MB-1 (CD37) underwent endocytosis at an intermediate rate, and 7.2 (class II) and 1F5 (CD20) were internalized slowly. Trichloroacetic acid precipitation and high-performance liquid chromatography revealed the following relative rates of ^{125}I-MoAb degradation: DA4-4 (μ) > HD6 (CD22) > HD37 (CD19) > G28-5 (CDw40) > MB-1 (CD37) > 1F5 (CD20) > 7.2 (class II).

INTRODUCTION

The identification of tumor-associated antigens has provided an approach by which malignant cells might be eradicated with minimal effects on adjacent normal cells. Preliminary promising results have been obtained in both animal and human tumor models using unmodified MoAbs[3] (1–3), radiolabeled antibodies (4, 5), and immunotoxins (6, 7). Malignancies of the B-lymphocyte lineage are ideal for evaluating the utility of antibody-targeted therapy because (*a*) B-cell surface antigens have been well characterized (8), (*b*) numerous high affinity B-cell specific antibodies are available, (*c*) human anti-mouse antibody formation is minimal in such patients (9), and (*d*) lymphomas have been shown to be sensitive test systems for new antineoplastic agents (10).

Criteria for selecting an antibody for immunoconjugate trials are poorly defined. Parameters believed to be important include the number of antigen sites per target cell, uniformity of antigen expression on all malignant cells, absence of binding to nontarget tissues, and the immunoreactivity, avidity, isotype, rate of internalization, intracellular routing, and rate of degradation of the targeting MoAb. In an attempt to critically evaluate the relative merits of a panel of MoAbs directed against different B-cell differentiation antigens, we have studied these parameters for MoAbs HD37 (anti-CD19), 1F5 (anti-CD20), HD6 (anti-CD22), MB-1 (anti-CD37), G28-5 (anti-CDw40), DA4-4 (anti-IgM), and 7.2 (anti-class II) on a cell line (Daudi) derived from a patient with Burkitt's lymphoma.

MATERIALS AND METHODS

Cell Suspensions. Malignant B-cell (Daudi) lines were maintained in log-phase growth in RPMI 1640 supplemented with 12% FCS, 2 mM glutamine, and 1 mM pyruvate at 37°C in 5% carbon dioxide. Fresh lymphoma cells were obtained from diagnostic biopsies performed on patients with B-cell lymphomas at University Hospital, Seattle after obtaining informed consent. Single cell suspensions were made by mincing lymph node fragments and pressing the cells through wire screens. After removing cell aggregates by sedimentation, cells were stored in 10% dimethyl sulfoxide/90% FCS in liquid nitrogen until analyzed.

Antibodies. MoAbs used in these experiments included anti-HLA class II MoAb 7.2 (IgG_{2a}), anti-human IgM (μ chain) MoAb DA4-4 (IgG_1, from the American Type Culture Collection), anti-CD19 MoAb HD37, and anti-CD22 MoAb HD6 (both IgG_1, gifts from Dr. Ellen Vitetta, University of Texas at Dallas, and Dr. Moldenhauer, German Cancer Research Center, Heidelberg), anti-CD20 MoAb 1F5 and anti-CDw40 MoAb G28-5 (both IgG_{2a}, gifts from Dr. Jeffrey Ledbetter, Oncogen Corporation, Seattle), anti-CD37 MoAb MB-1 (IgG_1, gift from Dr. Ron Levy, Stanford University), and control MoAbs 9E8 (IgG_{2a}), and MOPC-21 (IgG_1). The seven pan-B MoAbs studied were chosen because of the reliability of their expression on human B-cell malignancies (Table 1). MoAbs were purified from ascitic fluid by affinity chromatography on Sepharose-*Staphylococcal* protein A (11) or by ion-exchange chromatography. Protein concentrations were determined using the BCA assay following the recommendations of the manufacturer (Pierce Chemical Company, Rockford, IL).

Radioiodination. MoAbs were labeled with ^{125}I-Na by the Iodo-Gen method. MoAb (100 μg) was incubated with 0.5 mCi of ^{125}I-Na in glass tubes coated with 10 μg of Iodogen (Pierce Chemical Co., Rockford, IL) for 10 min at room temperature. Free ^{125}I-Na was removed by chromatography on a Pharmacia PD-10 column (Pharmacia, Piscataway, NJ). Eluted fractions containing ^{125}I-labeled antibody were pooled and stored at 4°C. The immunoreactivity (proportion of antibody molecules in a preparation which were capable of binding to antigen) and avidity of radiolabeled MoAbs were determined by modifications of previously described methods (12–14).

Determinations of Number of Cell Binding Sites. Two methods were used to estimate the number of binding sites for B MoAbs on Daudi cells. In the first method, serial dilutions of ^{125}I-labeled MoAbs were bound to a fixed number of Daudi cells (200,000) at room temperature (22°C) for 2 h. Cell suspensions were then centrifuged and the number of bound and free cpm quantitated by gamma counting. The ratio of bound/free cpm was plotted *versus* the concentration of antibody, and the *X*-intercept was taken as the number of antigen binding sites per cell (12–14). Data were corrected for the immunoreactivity of the MoAb (see above) and for nonspecific binding using irrelevant cell lines and irrelevant ^{125}I-labeled MoAbs as described by Badger *et al.* (13). Reanalysis of the data using the LIGAND program to model nonspecific binding as a fitted parameter yielded similar avidity constants (14).

In the second method, the numbers of cell surface binding sites were estimated by flow cytometry using fluorescent microbead standards

Received 1/10/89; revised 4/5/89, 5/22/89; accepted 6/8/89.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by NIH Grants CA-28149, CA-44991, and CA-41081 and by DOE Grant DE-FG06-88ER60719. During portions of this research, Dr. Press was the recipient of a First Independent Research and Training Award from the NIH (CA-46134).

[2] To whom requests for reprints should be addressed, at University Hospital Cancer Center, RC-08, University of Washington, Seattle, WA 98195.

[3] The abbreviations used are: MoAb, monoclonal antibody; FCS, fetal calf serum; FITC-GAMIg, fluorescein-conjugated goat anti-mouse immunoglobulin; IT, immunotoxin; SN, supernatant; TCA, trichloroacetic acid; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase.

Table 1 *Immunophenotypes of human B cell malignancies*[a]

Antigen expression	Non-Hodgkin's lymphoma	B-chronic lymphocytic leukemia	Hairy cell leukemia	Non-T, Non-B acute lymphocytic leukemia
CD19	352/419 (84%)	123/125 (98%)	18/30 (60%)	314/323 (97%)
CD20	401/441 (91%)	85/92 (92%)	23/23 (100%)	20/46 (43%)
CD22	327/421 (78%)	21/42 (50%)	6/6 (100%)	3/11 (27%)
CD37	212/228 (93%)	52/67 (78%)	11/11 (100%)	3/15 (20%)
CDw40	22/23 (96%)	24/31 (77%)	4/4 (100%)	N.A.
DR	427/438 (97%)	50/50 (100%)	7/9 (78%)	323/325 (99%)
sIg	336/349 (86%)	115/137 (84%)	26/27 (96%)	0/35 (0%)

[a] Compiled from references (8, 19–26).

(Flow Cytometry Standards Corporation, Research Trangle, NC). Briefly, 5×10^5 Daudi cells were stained to saturation with each of the study antibodies, washed twice, stained with 1:20 FITC-GAMIg, washed twice, and analyzed by flow cytometry. The fluorescence intensity per cell was interpolated onto a standard fluorescence calibration curve constructed using a Quantitative Fluorescein Microbead Standards Kit. Results were normalized using the fluorescence to protein (F/P) ratio determined empirically for each MoAb using Simply Cellular Microbeads (Flow Cytometry Standards Corp.) as described by the manufacturer for indirect immunofluorescence.

Microfluorimetry. The binding of MoAbs to target cells was determined by indirect immunofluorescence using a FITC-GAMIg (TAGO Inc., Burlingame, CA). Cells were incubated for 30 min on ice with MoAbs at saturating concentrations, washed, incubated with a 1:20 dilution of FITC-GAMIg, washed, and fixed with 1% paraformaldehyde. Cells were analyzed on a cell sorter (Facstar; Becton-Dickinson Inc., Sunnyvale, CA).

Endocytosis Assay. Daudi cells were washed in cold serum-free RPMI 1640 and pelleted by centrifugation. ^{125}I-labeled MoAbs were added to the cell pellets in a ratio of 1 μg protein (200,000 cpm) per 10^6 cells in a volume of 0.5 ml. Jurkat cells or irrelevant MoAbs 9E8 or MOPC21 were used as controls to assess nonspecific binding. Cells were incubated with ^{125}I-MoAbs at 4°C for 1 h and then washed twice with cold RPMI 1640. Aliquots containing 1×10^6 cells were plated in 200 μl RPMI 1640 in microtiter plates (Flow Laboratories), warmed to 37°C (in a humidified CO_2 incubator) for various time intervals, and then assayed for radioactivity in the SN, surface membrane, and intracellular compartments as described below.

Following 0, 1, 2, 4, 10, and 24 h of culture, cell suspensions were pelleted by centrifugation at 4°C at $300 \times g$ for 3 min. SNs were aspirated and the radioactivity was determined on a Beckman Gamma 7000 Counter (Palo Alto, CA). Surface-bound antibody was then removed from cell membranes using two consecutive 15 min acid/papain washes (15). The wash solution was prepared by titrating RPMI 1640 medium to pH 1.0 with 1 N HCl and adding 2.5 mg/ml papain (Sigma, St. Louis, MO). Cells were sedimented by centrifugation ($300 \times g$) and subjected to a second acid/papain wash. The two acid washes were pooled and the radioactivity in the eluted ^{125}I-MoAb was determined by gamma counting. This technique released ≥90% of the cell-associated radioactivity from Daudi cells labeled with antibodies DA4-4, 7.2, HD37, G28-5, 1F5, and MB-1 when cell suspensions were maintained at 0–4°C to inhibit endocytosis. (Only 70% of surface ^{125}I-HD6 (CD22) could be eluted under these control conditions. To facilitate comparison with the other antibodies, data for HD6 were normalized so that the cpm which could not be stripped from the cell surface at time 0 were subtracted from the total culture cpm.) Nonspecific binding of labeled MoAbs to cells lacking the target antigen represented <1% of the radioactivity bound to the cells expressing the antigen. After stripping of surface ^{125}I-MoAbs and collecting the acid washes, the cell pellets were harvested from microwells with cotton swabs. Cell pellets were assessed for the presence of internalized ("acid-resistant") ^{125}I-MoAbs by gamma counting.

Antibody Degradation. The extent of degradation of the ^{125}I-MoAbs was measured as described (15, 16). Culture SNs (0.2 ml) were mixed with 0.6 ml of 25% TCA to precipitate protein-bound ^{125}I released from the cell surface. Precipitates were washed with 0.5 ml 25% TCA, and the radioactivity in the pellets and washes was determined.

HPLC. SNs were harvested from cultures of ^{125}I-MoAb labeled Daudi cells after various time intervals (0, 4, 10, 24 h). Aliquots (50 μl) of crude SNs or the TCA soluble SN fractions (containing approximately 4000 cpm) were injected into a Beckman Model 338 HPLC apparatus fitted with a TSK4000SW gel filtration column, and a model 170 radioisotope detector. The retention times of the applied radioactive species were recorded and compared with standards (radiolabeled intact IgG, free ^{125}I, and ^{125}I-tyrosine). The distribution of radioactivity in fractions of different size ranges were plotted and the areas under the curves quantified by a dedicated analogue computer.

Immunoelectron Microscopy. Electron microscopy of immunoperoxidase-stained cells was employed to identify the intracellular compartments in which pan-B MoAbs were distributed. Attempts to use postembedding immunolabeling were unsuccessful due to the insensitivity of this technique in detecting cell-associated antibody at <400,000 molecules/cell. Consequently, pre-embedding immunolabeling with a monovalent Fab'-HRP-GAMIg reagent was performed. HRP (RZ = 3.0, Sigma, St. Louis, MO) was conjugated to Fab' fragments of GAMIg by the metaperiodate method (15, 17). Immunoperoxidase staining was performed by incubating 5×10^6 cells with a saturating concentration of MoAb for 30 min at 4°C, washing three times with RPMI 1640, incubating with HRP-Fab'-GAMIg for 30 min at 4°C, washing three times, and then warming cells to 37°C. Following 0–24 h of incubation, cells were washed, fixed for 30 min with half-strength Karnovsky's fixative, washed twice in 0.1 M cacodylate buffer, and reacted with 225 μl diaminobenzidine (0.5 mg/ml) (Sigma, St. Louis, MO) in 0.2 M Tris buffer (pH 7.4) containing 0.0009% hydrogen peroxide for 15 min. After two washes in 0.1 M cacodylate buffer, cells were postfixed in 1% osmium tetraoxide, and processed for electron microscopy. Between 30 and 60 cells (sectioned through the nucleus) per grid were scored for the presence of total surface label, for patching, capping, and the presence of labeled microinvaginations, endosomes, and lysosomes. Unstained sections were used to score cells as having peroxidase-positive surface membranes, endosomes, and lysosomes. Peroxidase-labeled organelles contrasted starkly with unlabeled cellular compartments in these uncounterstained sections. Organelles were scored as peroxidase-positive or negative in an "all or none" fashion. For photographic purposes, some grids were counterstained with uranyl acetate and lead citrate. Some fixed cell suspensions were treated by the method of Barka and Anderson (18) to identify acid phosphatase activity (a lysosomal marker) before embedding in Epon 812. Previous studies have shown that Fab'-HRP-GAMIg labeling by this technique does not alter the rate or route of immunoconjugate internalization (16).

RESULTS

Characterization of MoAbs. The binding avidities, immunoreactivities, and the number of surface binding sites for the MoAbs on Daudi cells are summarized in Table 2. The antibodies segregated into two groups with respect to surface antigen density: CD20, CD37, μ, and HLA class II molecules were highly expressed (200,000–400,000 binding sites/cell) whereas CD19, CD22, and CDw40 were less densely expressed (80,000–100,000 sites/cell). For comparison purposes, the expression of B-cell antigens on fresh lymphoma cells were quantified for five patients with low and intermediate grade B-cell lymphomas. Large numbers of binding sites were found for MoAbs 1F5 (CD20; 128,000 ± 33,000 sites) and MB-1 (CD37; 59,000 ± 12,000 sites) with lower antigen densities for the other MoAbs [DA4-4 (μ), 50,000 ± 27,000 sites; 7.2 (class II), 28,000 ± 6000 sites; G28-5 (CDw40), 19,000 ± 3000 sites; HD37

Table 2 *Characterization of murine mAbs recognizing [redacted] B cell antigens*

Antibody	Antigen[a]	Isotype	Binding sites/cell[b]	Avidity (L/M)	Immunoreactivity (%)[d]
DA4-4	μ	IgG1	330,772	4.34×10^9	92
HD6	CD22	IgG1	86,683	2.88×10^9	69
G28-5	CDw40	IgG2a	86,835	4.50×10^9	86
HD37	CD19	IgG1	91,131	0.30×10^9	76
MB-1	CD37	IgG1	234,311	2.99×10^9	92
1F5	CD20	IgG2a	306,097	0.37×10^9	59
7.2	Class II	IgG2b	393,163	1.71×10^9	74

[a] CD, cluster designation as assigned by the Third International Workshop on Monoclonal Antibodies (8).

[b] Means of 5 experiments (two performed with radiolabeled antibodies and three with the fluorescent microbead method). There was good correlation between the results obtained with the two different types of assays. (Standard errors of the means were <15% except for DA4-4 [±27%] and HD37 [±30%].)

[c] Avidities determined by Scatchard analyses of ^{125}I-MoAb binding to Daudi cells (14).

[d] Immunoreactivity determined by Lineweaver-Burk analyses of ^{125}I-MoAb binding to Daudi cells. (Average of 4 experiments [Standard Errors ≤8% except 7.2 = 15%].)

(CD19), 19,000 ± 200 sites; and HD6 (CD22), 8000 ± 4000 sites]. All seven MoAbs bound to 90–100% of Daudi cells with narrow, unimodal peaks on flow cytometry. Binding sites were also fairly uniformly present on fresh lymphoma B-cells for MoAbs 1F5 (CD20), 7.2 (class II), and MB-1 (CD37) (with 97–100%, 85–100%, and 76–99%, respectively, of the B-cells from the patients expressing the relevant antigen). However, there was more variability in antigen expression for MoAbs DA4-4 (μ), HD6 (CD22), HD37 (CD19), and G28-5 (CDw40) with 20–92%, 52–89%, 77–89%, and 77–93%, respectively, of fresh lymphoma B-cells bearing the relevant antigens.

Endocytosis and Degradation of Pan-B MoAbs. MoAbs differed substantially in their behavior after cell binding. DA4-4 (μ), HD6 (CD22), and HD37 (CD19) were rapidly internalized by cells (Fig. 1, *a*, *b*, and *d*) resulting in the disappearance of radioactivity from the cell surface and appearance of label in the intracellular compartment. Peak levels of intracellular ^{125}I-MoAb were detected at 4 h of culture with subsequent declines attributable to degradation and exocytosis of ^{125}I-MoAbs (see below). SN radioactivity appeared rapidly with these antibodies and accounted for 70–80% of total culture cpm at 24 h. G28-5 (CDw40) was also rapidly internalized, but intracellular retention of this MoAb was more prolonged, with 40% of the label remaining in an intracellular compartment between 4 and 24 h, and with a plateau in SN cpm at 30% (Fig. 1*c*). MoAbs MB-1 (CD37), 7.2 (class II), and 1F5 (CD20) were characterized by more prolonged retention on the cell surface with slower intracellular accumulation of label (Fig. 1, *e*, *f*, and *g*).

Fig. 2 shows that ^{125}I-DA4-4 (μ) was the MoAb most extensively degraded by Daudi cells, with 40–50% of all culture radioactivity being TCA soluble after 24 h of culture. ^{125}I-1F5 (CD20) and ^{125}I-7.2 (class II) showed the least degradation with <10% of the label being TCA-soluble after 24 h. These experiments demonstrated that the rapid disappearance of 40–45% of surface-bound 1F5 over the first 4 h of culture seen in Fig. 1*f* was primarily due to dissociation, a finding consistent with its low avidity.

Analysis of ^{125}I-MoAbs in Culture Supernatants using HPLC. Culture SNs were analyzed by HPLC using a size exclusion column to determine the size of molecules bearing ^{125}I. In cultures containing rapidly internalized and degraded antibodies, relatively little radioactivity in the SN was on intact IgG molecules after 24 h [*e.g.*, 32% for ^{125}I-DA4-4 (anti-μ)]. In contrast, the majority of SN cpm in cultures containing slowly degraded antibodies was present on intact IgG after 24 h of incubation (*e.g.*, 67% for MoAb 7.2). HPLC confirmed the efficacy of TCA in precipitating intact ^{125}I-MoAb molecules; all TCA soluble radioactivity in SNs was present on molecular species ≤2,000 Daltons (data not shown).

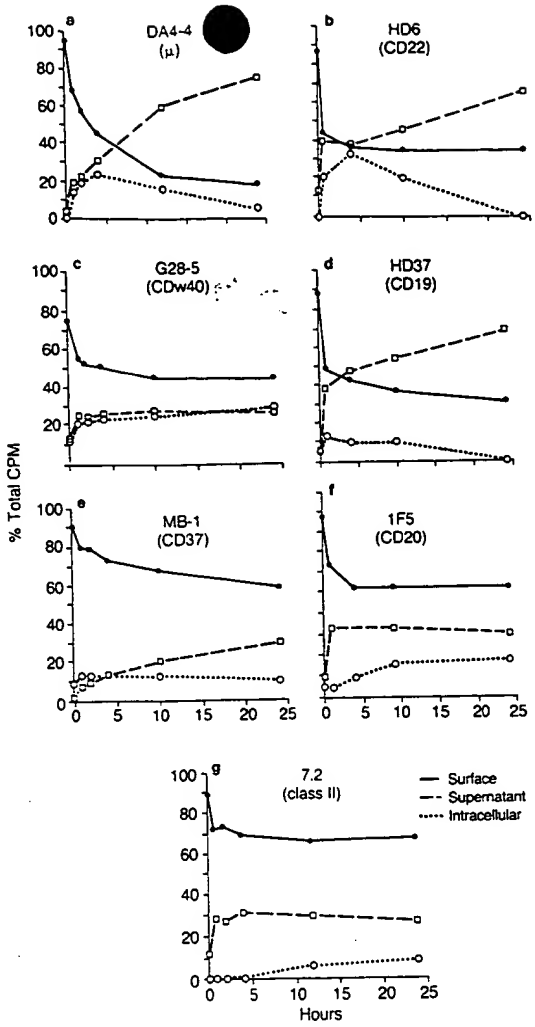


Fig. 1. Endocytosis of ^{125}I-labeled MoAbs by Daudi cells. Results are expressed as the percentage of total radioactivity on the cell surface (released by acid-papain, ●), inside cells (not released by acid-papain, O) and in culture supernatants (TCA soluble + TCA precipitable cpm) (□). Seven other experiments yielded similar results.

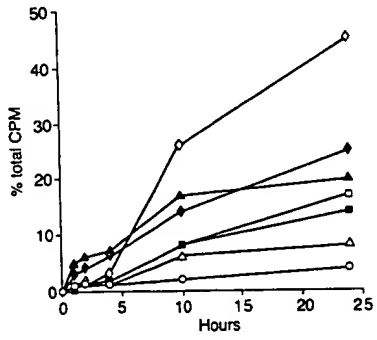


Fig. 2. Degradation of ^{125}I-labeled MoAbs by Daudi cells. To assess the rates at which ^{125}I-MoAbs were degraded and exocytosed, SNs from the experiment shown in Fig. 1 were precipitated with 25% TCA, centrifuged, decanted, and TCA soluble cpm quantitated by gamma counting. ◇, DA4-4 (μ); ◆, HD6 (CD22); ▲, HD37 (CD19); □, G28-5 (CDw40); ■, MB-1 (CD37); △, 1F5 (CD20); O, 7.2 (Class II).

Immunoelectron Microscopy of MoAb Internalization. Endocytosis and intracellular trafficking of MoAbs was demonstrated directly by immunoperoxidase electron microscopy. All cells showed circumferential labeling at time 0 [shown for MoAb HD6 (CD22) in Fig. 3*a*]. MoAbs DA4-4 (μ), HD-6

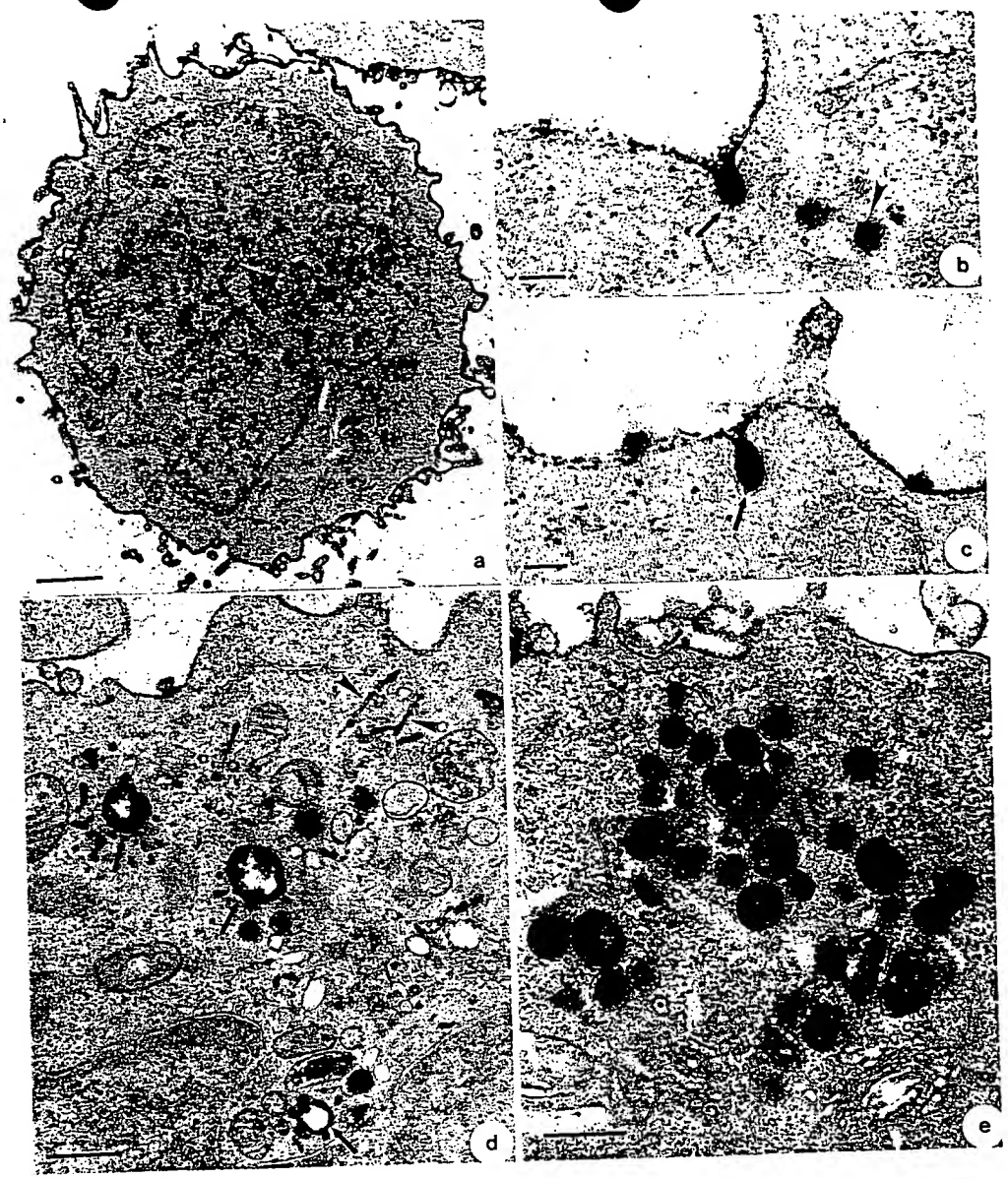


Fig. 3. Immunoperoxidase electron micrography of Daudi cells labeled with MoAb HD6 (CD22). Cells were labeled with MoAb HD6 and peroxidase-conjugated Fab'-GAMIg at 4°C before warming cells to 37°C to initiate endocytosis. At time 0, cells displayed circumferential surface staining (*a*) (*bar*, 1 μm). Within minutes of warming at 37°C extensive endocytosis of HD6 via "coated pits" (*arrows* in *b* and *c*) and "coated" vesicles (*arrowhead* in *b*) was apparent (*bars*, 0.1 μm). Within 30 min cells contained numerous peroxidase-labeled endocytic vesicles or "receptosomes" (*arrows* in *d*) and tubular cisternae (*arrowheads* in *d*) (bar, 0.5 μm). Between 2 and 18 h after warming to 37°C, progressive accumulation of peroxidase in lysosomes was observed (*e*) (*bar*, 0.5 μm).

(CD22), and G28-5 (CDw40) were rapidly internalized via coated pits [shown for MoAb HD6 (CD22) in Fig. 3, *b* and *c*), which subsequently pinched off, giving rise to labeled endocytic vesicles (Fig. 3*d*). Within 30 min at 37°C, 65–70% of cells treated with these three MoAbs had detectable label in endosomes (Fig. 4, *a–c*). Subsequently, there was a slowly progressive increase in the percentage of cells containing labeled lysosomes [up to 67% for DA4-4 (μ), 40% for HD6 (CD22), and 37% for G28-5 (CDw40) after 18 h] (Figs. 3*e* and 4) accompanied by a slight decline in the percentage of cells containing labeled endosomes. MoAbs 1F5 (CD20) and 7.2 (class II) were much more slowly internalized as demonstrated by the prolonged persistence of surface label and the more gradual appearance of peroxidase label in endosomes and lysosomes (≥93% of cells retained surface label even after 18 h of culture (Fig. 4, *f* and *g*). Of note was the fact that cells labeled with MoAbs HD37 (CD19), MB-1 (CD37), 1F5 (CD20), and 7.2 (class II) showed progressive accumulation of label in the endosomes over the full 18-h time period studied (Fig. 4, *d–g*), in contrast to the plateau and decline seen with MoAbs DA4-4 (μ), HD6 (CD22), and G28-5 (CDw40) (Fig. 4, *a–c*). Furthermore, MoAbs DA4-4 (μ), HD6 (CD22), and G28-5 (CDw40) were found to enter a prominent tubular endocytic compartment (*arrowheads* in Fig. 3*d*), whereas the other four antibodies had very limited transport to this compartment (data not shown). These tubular cisternae were frequently seen in close proximity to the trans-Golgi apparatus, which has been reported to be important in translocation of toxins such as ricin and diphtheria toxin to the cytosol (27). The main Golgi apparatus itself was not prominently involved in the intracellular trafficking of any of the seven MoAbs studied.

It is important to note that the two assays used in Figs. 1 and 4 measure different parameters, and therefore the contours of the curves obtained differ. The radioimmunoassays depicted in Fig. 1 give a quantitative measure of the relative proportions of antibody on the cell surface, inside cells, and in the culture supernatant at various time points. For any given time in Fig. 1, the sum of the values in these three compartments is 100%. In contrast, the immunoelectron microscopy studies in Fig. 4 measure the percentage of cells at each time point which have any amount of antibody on their surface, in endosomes, and in lysosomes. This technique does not measure the relative distribution of antibody in the various compartments, hence, the values for time points later than time 0 in Fig. 4 do not add up to 100%. Thus, after 2 h of incubation, almost 50% of the ^{125}I-DA4-4 cpm had been cleared from the surface membrane (Fig. 1*a*), but 90% of cells examined ultrastructurally still retained small amounts of surface bound antibody (Fig. 4*a*).

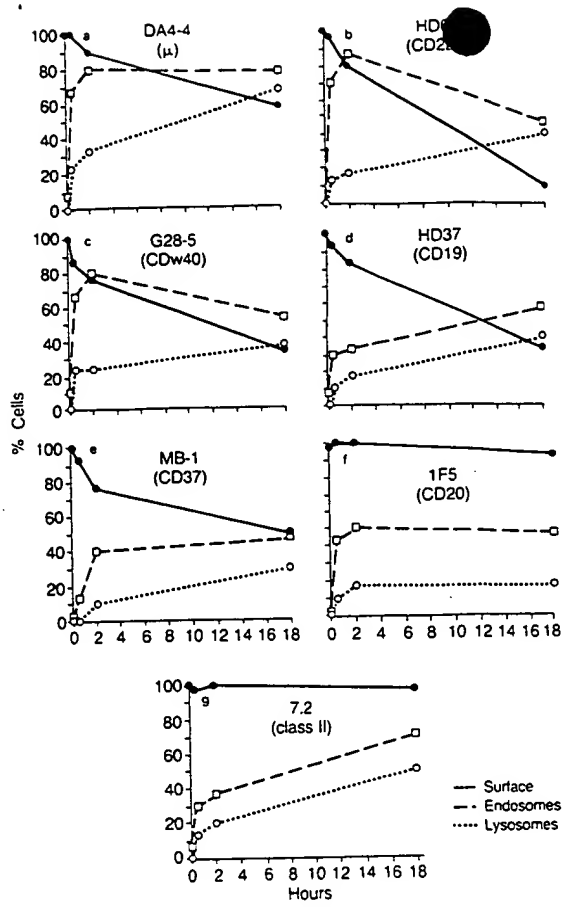


Fig. 4. Endocytosis of MoAbs by Daudi cells as determined by immunoelectron microscopy. Cells were treated with MoAbs and peroxidase-conjugated Fab'-GAMIg at 4°C as described in the legend for Fig. 3. At 0, 0.5, 2, and 18 h cells were washed, fixed and processed for electron microscopy. Cells were scored for peroxidase-positive surface membranes (●), endocytic vesicles (□), and lysosomes (○) morphometrically.

Electron microscopy revealed that the density of surface label remaining on cells after 18 h varied greatly. MoAb DA4-4 (μ) was distributed in small surface patches (Fig. 5*a*), whereas cells labeled with MoAbs 1F5 (CD20) and 7.2 (class II) often showed dense circumferential distributions even after 18 h (Fig. 5*b*). The slow rate of internalization of MoAb 7.2 (Class II) by Daudi cells (Figs. 1 and 4) was quite a surprise, since it had previously been shown that normal B-cells spontaneously internalize Class II molecules ([redacted]) and we expected crosslinking with MoAb 7.2 to accelerate this process. However, MoAb 7.2 proved to be the slowest of the seven MoAbs cleared from the surface of Daudi cells. This finding may reflect rapid recycling of intact MoAb 7.2 back to the surface membrane after endocytosis (28), or slow modulation of class II antigen, which has been reported to occur with some malignant B-cells (29).

DISCUSSION

Several groups have assessed the merits of various pan-B antibodies for therapy of B-cell malignancies (1, 4, 5, 19, 30, 31), but MoAb selection has remained arbitrary because the relative importance of the various parameters influencing efficacy remains uncertain. This report compares the *in vitro* immunobiological properties of seven MoAbs which have potential clinical utility. Table 3 summarizes the salient features of the seven MoAbs studied. Since different MoAbs targeting the same surface antigen can vary greatly in their rates of endocytosis and subsequent intracellular routing (16, 32), caution should be exerted in extrapolating the findings reported for the MoAbs studied in this report to other MoAbs or to other cell types, even if the same surface antigen is targeted.

Of the parameters considered desirable for antibody-directed immunotherapy, high surface antigen density is perhaps least controversial since it permits a high concentration of effector molecules to be focused on target cells. High antibody avidity also appears desirable based on *in vitro* IT studies (31), though moderate or low avidity MoAbs may "percolate" deeper into solid tumor masses *in vivo* (33). High immunoreactivity may maximize targeting and minimize toxicity in IT and radioimmunotherapy trials, but MoAbs with moderately low immunoreactivity may suffice for unmodified antibody trials (3) since toxicity is minimal and dose escalatation can compensate for a reduced immunoreactive fraction. Antibody isotype appears to be of primary importance for trials using unmodified antibodies, where the murine IgG_3 and IgG_{2a} isotypes are most effective at interacting with human effector cells (34, 35). Rapid MoAb internalization into target cells is deleterious for unmodified antibody trials [because interactions with effector cells are abrogated (36–38)], advantageous for immunotoxin studies [since the reagents must reach ribosomes (6)], and isotope-dependent for radioimmunoconjugate protocols [since intracellular dehal-

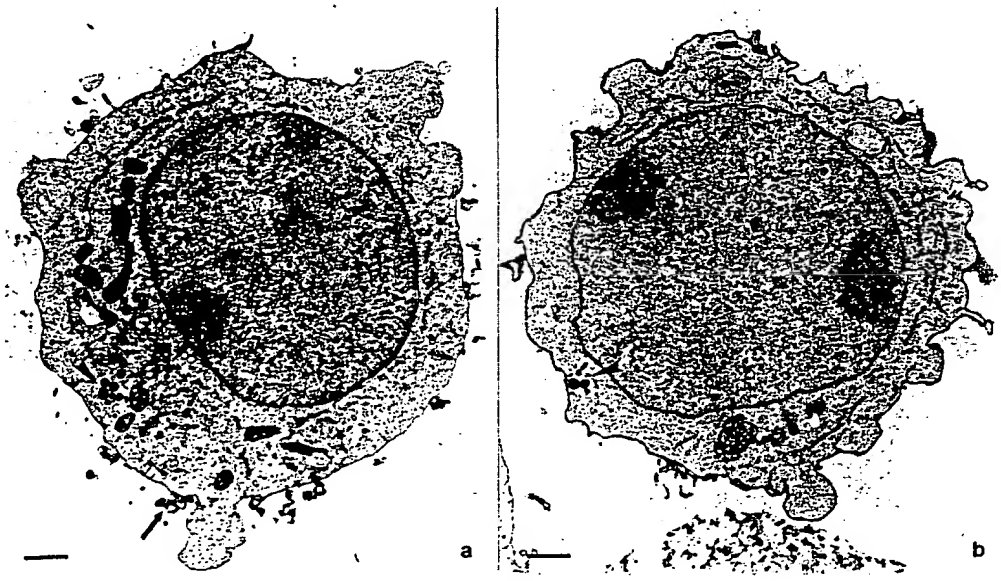


Fig. 5. Variability in retention of surface antibody on Daudi cells labeled with MoAb DA4-4 (μ) or MoAb 7.2 (class II). Immunoelectron micrographs of cells cultured for 18 h at 37°C after surface labeling with either MoAb DA4-4 (*a*) or MoAb 7.2 (*b*) and HRP-Fab'-GAMIg. DA4-4-labeled cells retaining surface label had only small patches of residual surface antibody (*arrow* in *a*), whereas MoAb 7.2-labeled cells had dense circumferential residual surface label (*b*). Cells in both (*a*) and (*b*) had some internalized antibody in lysosomes (*arrowheads*). (*Bars*, 1 μm).

Table 3 *Summary of pan B antibody characteristics*[a]

Antibody (antigen)	DA4-4 (μ)	HD6 (CD22)	G28-5 (CDw40)	HD37 (CD19)	MB-1 (CD37)	1F5 (CD20)	7.2 (Class II)
Antigen density	+++	+	++	++	+++	+++	+++
Avidity	+++	+++	+++	++	+++	++	+++
Immunoreactivity	++++	+++	++++	+++	++++	++	+++
Extent of endocytosis	++++	++++	+++	++	++	+	+
Surface retention	+	+	+	++	++	+++	+++
Degradation rate	++++	+++	++	++	++	+	+
Tubulocisternal delivery[b]	++++	+++	++	+	−	−	+

[a] Determined on Daudi cells.
[b] Delivery of antibody to "tubular cisternae" in cytoplasm (see text).

ogenation of ^{131}I-MoAbs is detrimental (4), but intracellular localization of short acting isotopes (*e.g.*, α-emitters) is beneficial]. Intracellular routing of ITs to the trans-Golgi reticulum (27) and prolonged retention in prelysosomal compartments may maximize IT efficacy (16, 27), whereas rapid delivery of ITs and radioimmunoconjugates to lysosomes may attentuate their clinical efficacy (16, 39).

Cross-reactivity of MoAbs with nonmalignant tissues increases the potential toxicity of immunotherapy, though temporary elimination of normal, circulating B-cells by pan-B MoAbs has been well tolerated (3, 4). Cross-reactivity of MoAbs with nonlymphoid cells or tissues (*e.g.*, kidney, nerve axons) may lead to unsuspected toxicities (40), although some degree of normal tissue cross-reactivity is tolerable as shown by trials using anti-CD37, antiferritin, and anti-class II antibodies (4, 29, 41). Interestingly, cross-reactivity of anti-CDw40 antibodies such as G28-5 with carcinomas may actually increase their clinical utility (8).

Since many B-cell antigens are receptors for naturally occurring ligands (8, 19), it is possible that MoAbs targeting these molecules may induce or suppress differentiation, activation or mitogenesis of tumor B-cells. However, we have seen no convincing evidence to support this concern in the patients we have treated so far with anti-B-cell MoAbs (3, 4). Antigen shedding is an undesirable feature since circulating antigen impedes MoAb targeting to tumor sites (1) and may result in immune complex disease.

On the basis of the parameter analysis above, we speculate that MoAbs 1F5 (CD20) and MB-1 (CD37) may be well suited for *in vivo* applications as unmodified antibodies since they are cleared from the surface of cells less rapidly than HD6 (CD22), DA4-4 (μ), and G28-5 (CDw40), have more surface binding sites than HD37 (CD19), HD6 (CD22), and G28-5 (CDw40), and will not cross-react with nontarget cells as extensively as MoAb 7.2 (class II). MoAb 1F5 (CD20) might be somewhat preferable because of its higher specificity for B-cells, higher number of surface binding sites, slower endocytosis and degradation rates, and IgG_{2a} isotype. Conversely, MoAb MB-1 (CD37) has a higher immunoreactivity (4) and possibly less chance of inducing activation and proliferation of B-cells (8).

Selection criteria for MoAbs used as carriers of drugs or toxins differ from those for antibodies used in unmodified form. Ideally, MoAbs to be used as drug or toxin conjugates should be efficiently delivered to target cells, rapidly internalized, and delivered to endocytic compartments [*e.g.*, receptosomes or trans-Golgi reticulum (27)]. We would predict that HD6 (CD22) and G28-5 (CDw40) might make effective immunotoxins because of their rapid internalization by malignant B-cells. Anti-μ MoAbs (*e.g.*, DA4-4) are less desirable for *in vivo* IT trials because of the high concentrations of IgM in blood, even though they are rapidly endocytosed by cells and make effective ITs *in vitro*. MoAb 1F5 (CD20) is poorly internalized, and would not be expected to make a good immunotoxin reagent.

MoAb MB-1 (CD37) is a good candidate for radioimmunotherapy trials because of its high avidity, high immunoreactivity, large number of surface binding sites, and intermediate rate of dehalogenation. MoAb 1F5 (CD20) may also suffice for radioimmunotherapy because of its slow dehalogenation and the high number of binding sites on B-cell tumors, though its low immunoreactivity is unfavorable. The rapid degradation rates for MoAbs DA4-4 (μ) and HD6 (CD22) make these antibodies less attractive as candidates for trials using radioiodinated MoAbs. The reactivity of MoAb 7.2 (class II) with many non-B-cells is undesirable, though animal and human experiments suggest that this may not be an absolute impediment (5, 29).

These predictions have not yet been prospectively tested, but the available evidence is supportive for our hypotheses. MoAb 1F5 (CD20) in unmodified (or trace-labeled) form induced temporary responses in three of five lymphoma patients [one minor, one partial, and one complete response (3, 4)], MoAb HD6 (CD22) made a much more potent ricin A-chain IT than did HD37 (CD19) (32, 42), anti-CD20 MoAbs made totally ineffective ITs (43), and ^{131}I-labeled MoAb MB-1 (CD37) induced major responses in all five patients with refractory lymphomas treated [four complete responses and one partial response (4)].

ACKNOWLEDGMENTS

We are grateful to Drs. E. Vitetta, J. Ledbetter, G. Moldenhauer, and R. Levy for providing antibodies, to Dr. E. Vitetta for reviewing the manuscript, and to Judy Groombridge and Liz Caldwell for expert technical assistance.

REFERENCES

1. Meeker, T. C., Lowder, J., Maloney, D. G., Miller, R. A., Thielemans, K., Warnke, R., and Levy, R. A clinical trial of anti-idiotype therapy for B cell malignancy. Blood, *65:* 1349–1363, 1985.
2. Bernstein, I. D., Tam, M. R., and Nowinski, R. C. Mouse leukemia: therapy with monoclonal antibodies against a thymus differentiation antigen. Science (Wash. DC), *207:* 68–71, 1979.
3. Press, O. W., Appelbaum, F., Ledbetter, J. A., Martin, P., Zarling, J., Kidd, P., and Thomas, E. D. Monoclonal antibody IF5 (anti-CD-20) serotherapy of human B. Cell lymphomas. Blood, *69:* 584–591, 1987.
4. Press, O. W., Eary, J., Badger, C., Martin, P. J., Appelbaum, F., Levy, R., Miller, R., Brown, S., Nelp, W. B., Krohn, K. A., Fisher, D., DeSantes, K., Porter, B., Kidd, P., Thomas, E. D., and Bernstein, I. D. Treatment of refractory non-Hodgkin's lymphoma with radiolabeled MB-1 (anti-CD37) antibody. J. Clin. Oncol., in press, 1989.
5. DeNardo, S. J., DeNardo, G. L., O'Grady, L. F., Levy, N. B., Mills, S. L., Macey, D. J., McGahan, J. P., Miller, C. H., and Epstein, A. L. Pilot studies of radioimmunotherapy of B cell lymphoma and leukemia using I-131 Lym-1 monoclonal antibody. Antibody Immunoconj. Radiopharm., *1:* 17–33, 1988.
6. Vitetta, E. S., and Uhr, J. W. The potential use of immunotoxins in transplantation, cancer therapy, and immunoregulation. Transplantation, *37:* 535–538, 1984.
7. Pastan, I., Willingham, M. C., and FitzGerald, D. G. Immunotoxins. Cell, *47:* 641–647, 1986.
8. McMichael, A. J. Leukocyte Typing III, pp. 302–363 & 432–469, Oxford, England, Oxford University Press, 1987.
9. Shawler, D. L., Bartholomew, R. M., Smith, L. M., and Dillman, R. O. Human immune response to multiple injections of murine monoclonal IgG. J. Immunol., *135:* 1530–1535, 1985.
10. DeVita, V. T. Principles of Chemotherapy. *In:* V. T. DeVita, S. Hellman, and S. A. Rosenberg (ed.), Cancer: Principles and Practice of Oncology, pp. 257–284. Philadelphia: J. B. Lippincott, 1985.
11. Ey, P., Prowse, S., and Jenkin, C. Isolation of pure IgG_1, IgG_{2a} and IgG_{2b} immunoglobulins from mouse serum using protein A Sepharose. Immunochemistry, *15:* 420–436, 1978.
12. Lindmo, T., Boven, E., Cuttitta, F., Fedorko, J., and Bunn, P. A., Jr. Determination of immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. J. Immunol. Methods, *72:* 77–89, 1984.
13. Badger, C. C., Krohn, K. A., and Bernstein, I. D. *In vitro* measurement of

avidity of radioiodinated antibodies. Nucl. Med. Biol., 605–610, 1987.
14. Munson, P. J., and Rodbard, D. LIGAND: A versatile computerized approach for characterization of ligand-binding systems. Analyt. Biochem., *107:* 220–239, 1980.
15. Press, O. W., Hansen, J. A., Farr, A., and Martin, P. J. Endocytosis and degradation of murine anti-human CD3 monoclonal antibodies by normal and malignant T lymphocytes. Cancer Res., *48:* 2249–2257, 1988.
16. Press, O. W., Martin, P. J., Thorpe, P. E., and Vitetta, E. S. Ricin A-chain containing immunotoxins directed against different epitopes on the CD2 molecule differ in their ability to kill normal and malignant T cells. J. Immunol., *141:* 4410–4417, 1988.
17. Farr, A., and Nakane, P. Immunohistochemistry with enzyme labeled antibodies: a brief review. J. Immunol. Methods, *47:* 129–144, 1981.
18. Barka, T., and Anderson, P. Histochemical methods for acid phosphatase using hexaonium paraosanilin as coupler. J. Histochem. Cytochem., *10:* 741–753, 1962.
19. Uckun, F. M., Jaszcz, W., Ambrus, J. L., Fauci, A. S., Gajl-Peczalska, K., Song, C. W., Wick, M. R., Myers, D. E., Waddick, K., and Ledbetter, J. A. Detailed studies on expression and function of CD19 surface determinant by using B43 monoclonal antibody and the clinical potential of anti-CD19 immunotoxins. Blood, *71:* 13–29, 1988.
20. Aisenberg, A. C., Wilkes, B. M., Long, J. C., and Harris, N. L. Cell surface phenotype in lymphoproliferative disease. Am. J. Med., *68:* 206–212, 1980.
21. Stein, R. S., Cousar, J., Flexner, J. M., and Collins, R. D. Correlations between immunologic markers and histopathologic classifications: clinical implications. Semin. Oncol., *7:* 244–253, 1980.
22. Pezutto, A., Dorken, B., Feller, A., Moldenhauer, G., Schwartz, R., Wernet, P., Thiel, E., and Hunstein, W. HD37 monoclonal antibody: a useful reagent for further characterization of "Non-T, Non-B" lymphoid malignancies. *In:* E. L. Reinherz, B. F. Haynes, L. M. Nadler, and I. D. Bernstein (eds.), Leukocyte Typing II, Vol. 2, pp. 391–402. New York: Springer Verlag, 1986.
23. Link, M. P., Bindl, J., Meeker, T. C., Carswell, C., Doss, C. A., Warnke, R. A., and Levy, R. A unique antigen on mature B cells defined by a monoclonal antibody. J. Immunol., *137:* 3013–3018, 1986.
24. Pullen, J. D., Falletta, J. M., Crist, W. M., Vogler, L. B., Dowell, B., Humphrey, G. B., Blackstock, R., van Eys, J., Cooper, M. D., Metzgar, R. S., and Meydrech, E. F. Southwest oncology group experience with immunological phenotyping in acute lymphocytic leukemia of childhood. Cancer Res., *41:* 4802–4809, 1981.
25. Melo, J. V., San Miguel, J. F., Moss, V. E., and Catovsky, D. The membrane phenotype of hairy cell leukemia: a study with monoclonal antibodies. Semin. Oncol., *11:* 381–385, 1984.
26. Tubbs, R. R., Savage, R. A., Sebek, B. A., Fishleder, A., and Weick, J. K. Antigenic phenotype of splenic hairy cells. Am. J. Med., *76:* 199–205, 1984.
27. Olsnes, S., and Sandvig, K. How protein toxins enter and kill cells. *In:* A. E. Frankel (ed.), Immunotoxins, pp. 39–73. Boston: Kluwer Academic Publishers, 1988.
28. Pernis, B. Internalization of lymphocyte membrane components. Immunol. Today, *6:* 45–49, 1985.
29. Bridges, S. H., Kruisbeek, A. M., and Longo, D. L. Selective *in vivo* antitumor effects of monoclonal anti-I-A antibody on B cell lymphoma. J. Immunol., *139:* 4242–4249, 1987.
30. Letvin, N. L., Zalutsky, M. R., Lambert, J. M., Chalifoux, L. V., Nadler, L. M., Schlossman, S. F., and Al[illegible]s, H. L. Use of radiolabeled monoclonal anti-B1 antibody for B lymphocyte imaging in rhesus monkeys. Nucl. Med. Biol., *14:* 99–105, 1987.
31. May, R. D., Vitetta, E. S., Moldenhauer, G., and Dorken, B. Selective killing of normal and neoplastic human B cells with anti-CD19- and anti-CD22-ricin A chain immunotoxins. Cancer Drug Delivery, *3:* 261–272, 1986.
32. Letvin, N. L., Ritz, J., Guida, L. J., Yetz, J. M., Lambert, J. M., Reinherz, E. L., and Schlossman, S. F. *In vivo* administration of lymphocyte-specific monoclonal antibodies in nonhuman primates. I. Effects of anti-T11 antibodies on the circulating T cell pool. Blood, *66:* 961–966, 1985.
33. Weinstein, J. N., Eger, R., Covell, D., Black, C. D., Mulshine, J., Carrasquillo, J. A., Larson, S. M., and Keenan, A. M. The pharmacology of monoclonal antibodies. Ann. NY Acad. Sci., *507:* 199–210, 1987.
34. Anasetti, C., Martin, P. J., Morishita, Y., Badger, C. C., Bernstein, I. D., and Hansen, J. A. Human large granular lymphocytes express high affinity receptors for murine monoclonal antibodies of the IgG3 subclass. J. Immunol., *138:* 2979–2981, 1987.
35. Herlyn, D., Lubeck, M., Steplewski, Z., and Kowprowski, H. Destruction of human tumors by IgG2a monoclonal antibodies and macrophages. *In:* R. A. Reisfeld and S. Sell (eds.), Monoclonal Antibodies and Cancer Therapy, pp. 165–172. New York: Alan R. Liss Inc., 1985.
36. Ritz, J., Pesando, J. M., Sallan, S. E., Clavell, L. A., Notis-McConarty, J., Rosenthal, P., and Schlossman, S. F. Serotherapy of acute lymphoblastic leukemia with monoclonal antibody. Blood, *58:* 141–152, 1981.
37. Foon, K. A., Schroff, R. W., Bunn, P. A., Mayer, D., Abrams, P. G., Fer, M., Ochs, J., Bottino, G. C., Sherwin, S. A., Carlo, D. J., Herberman, R. B., and Oldham, R. K. Effects of monoclonal antibody therapy in patients with chronic lymphocytic leukemia. Blood, *64:* 1085–1093, 1984.
38. Dillman, R. O., Shawler, D. L., Dillman, J. B., and Royston, I. Therapy of chronic lymphocytic leukemia and cutaneous T-cell lymphoma with T101 monoclonal antibody. J. Clin. Oncol., *2:* 881–891, 1984.
39. Press, O., Vitetta, E., Farr, A., Hansen, J., and Martin, P. Evaluation of ricin A chain immunotoxins directed against human T cells. Cell. Immunol., *102:* 10–20, 1986.
40. Gould, B., Borowitz, M., Carter, P., Hertler, A., Moore, J. O., Renckman, W., Groves, E. S., Marafino, B., and Frankel, A. A phase I study of continuous infusion immunotoxin for refractory metastatic breast cancer, International Symposium on Immunotoxins. Durham, NC, p. 52, 1988.
41. Order, S. E., Stillwagon, G. B., Klein, J. L., Leichner, P. K. Siegelman, S. S., Fishman, E. K., Ettinger, D. S., Haulk, T., Kopher, K., Finney, K., Surdyke, M., Self, S., and Leibel, S. Iodine-131 antiferritin, a new treatment modality in hepatoma: a radiation therapy oncology group study. J. Clin. Oncol., *3:* 1573–1582, 1985.
42. Ghetie, M., May, R. D., Till, M., Uhr, J. W., Ghetie, V., Knowles, P. P., Relf, M., Brown, A., Wallace, P. M., Janossy, G., Amlot, P., Vitetta, E. S., and Thorpe, P. E. Evaluation of Ricin A chain-containing immunotoxins directed against CD19 and CD22 antigens on normal and malignant human B-cells as potential reagents for in vivo therapy. Cancer Res., *48:* 2610–2617, 1988.
43. Lambert, J., Senter, P., Yau-Young, A., Blattler, M., and Goldmacher, V. Purified immunotoxins that are reactive with human lymphoid cells. J. Biol. Chem., *260:* 22, 1985.

[CANCER RESEARCH 47, 5954–5959, November 15, 198

Antibody-directed Targeting of Liposomes to Human Cell Lines: Role of Binding and Internalization on Growth Inhibition[1]

Neil Berinstein,[2] Katherine K. Matthay,[3] Demetrios Papahadjopoulos, Ronald Levy, and Branimir I. Sikic[4]

Oncology Division, Department of Medicine, Stanford University School of Medicine, Stanford 94305 [N. B., R. L., B. I. S.], and Departments of Pharmacology [D. P.], Pediatrics [K. K. M.], and Cancer Research Institute [K. K. M., D. P.], University of California, San Francisco, School of Medicine, San Francisco, California 94143

ABSTRACT

Small unilamellar liposomes containing methotrexate or methotrexate-γ-aspartate were conjugated to *Staphylococcus aureus* protein A and were thus able to bind cell-specific immunoglobulins for targeting to malignant human B- and T-cell lines. We were able to demonstrate enhanced protein A liposome uptake and growth inhibition by targeting with an anti-major histocompatibility complex class II antibody recognizing two different B-cell lines. The enhanced growth inhibition was specific for the targeting antibody and amounted to a 2- to 3-fold lowering of the concentration of drug required to inhibit cell growth by 50% as compared to nontargeted liposomes or liposomes targeted with an antibody not recognizing a cell surface antigen. A strong association between enhanced growth inhibition and liposome internalization as assessed by fluorescent-activated cell sorter analysis of carboxyfluorescein containing protein A liposomes was seen. By contrast, specific enhancement of growth inhibition was not seen with several anti-idiotype antibodies or antibodies to T-cell differentiation antigens. Liposome internalization did not occur with these antibodies. Failure of growth inhibition and PA liposome internalization could not be explained by differences in cell binding of the antibody PA liposomes or the degree of protein A binding of the targeting antibody. Although the ability of the targeting antibody to bind to the cell and to protein A are important, these factors alone are not sufficient to guarantee internalization and growth inhibition. Variations in rates of internalization of various cell surface antigen-antibody complexes may account for different protein A liposome mediated cytotoxicities.

INTRODUCTION

Liposomes, containing chemotherapeutic agents, provide the possibility of selective specific drug delivery and cytotoxicity (1). Methods of conjugating proteins (2–4) have enabled the attachment of immunoglobulins or protein A to liposomes for specific targeting. Protein A has the property of binding preferentially certain isotypes of immunoglobulins (IgG2a and IgG2b) and immunoglobulins of certain species (rabbit and murine *versus* goat which binds less well) (5). The conjugation of cell-specific antibodies to liposomes containing chemotherapeutic agents has been shown to increase cytotoxicity selectively in several murine tumor cell lines (6, 7). However, there are only a few reports in the literature examining the application of liposome technology to human cell lines (8). Specific enhanced tumor cytotoxicity could be of clinical relevance, especially in the area of tumor purging of bone marrows for autologous transplantation. In this report, we evaluate the efficacy of monoclonal antibody-targeted PA liposomes[5] containing methotrexate and methotrexate-γ-aspartate in human neoplastic lymphoid cell lines.

Received 2/27/87; revised 8/10/87; accepted 8/18/87.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by NIH Grants CA 33303, CA 33399, CA 34233, and CA 39448.

[2] Research Fellow of the National Cancer Institute of Canada.

[3] Recipient of the American Cancer Society Junior Faculty Clinical Fellowship and University of California, San Francisco, Faculty Development awards.

[4] To whom requests for reprints should be addressed, at Division of Oncology (M211), Stanford University Medical Center, Stanford, CA 94305.

[5] The abbreviations used are: PA liposome, protein A conjugated to small unilamellar vesicles; FACS, fluorescent-activated cell sorter; MHC, major histocompatibility complex; IC_{50}, drug concentration that inhibits cell growth by 50%; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

MATERIALS AND METHODS

Liposomes. A 10:5:1 molar mixture of phosphatidylcholine, cholesterol, and *N*-4-(*p*-maleimidophenyl)butyryl phosphatidylethanolamine was suspended in a solution of 50 mM methotrexate-γ-aspartate as previously described (9). The suspension was sonicated for 1 h in a bath sonicator (Laboratory Supplies, Hicksville, NY). Any remaining large multilamelar liposomes were removed by centrifugation in an Eppendorf 3200 centrifuge for 30 min, and unencapsulated drug was removed by gel filtration on Sephadex G-75. The vesicles were then conjugated for 18 h at 25°C with thiolated *Staphylococcus aureus* protein A in isotonic 50 mM 2(*N*-morpholino)ethanesulfonic acid-50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-2 mM EDTA buffer, pH 6.7. The protein and lipid concentrations during conjugation were 0.25 g/liter and 2.6 mM, respectively. The conjugated vesicles were separated from unbound protein by flotation on a metrizamide gradient as described (8). Liposomes were sterilized by passing them through a 0.2-μm polycarbonate filter, and analyzed for lipid, protein, and drug content as previously described (10). Methotrexate PA liposomes were made in a similar fashion but with a 5:5:1 ratio of the three lipids suspended in a methotrexate concentration of 25 mM.

Carboxyfluorescein PA liposomes were prepared as above but with the lipids suspended in a solution of 100 mM carboxyfluoroscein instead of methotrexate-γ-aspartate.

Cell Lines. Cells used included the human T-cell leukemia line CEM/VLB (obtained from Dr. W. T. Beck, Memphis, TN), the human B-cell lymphoma lines TAB (obtained from Dr. S. Smith, Stanford, CA) (11), OCI LY8 (obtained from Dr. H. Messner, Toronto, Ontario, Canada) (12), and the murine lymphoma line AKR/J SL2 (obtained from Dr. I. Bernstein, Seattle, WA).

Antibodies. Antibodies for targeting experiments included the anti-CD5 antibody Leu-1 (IgG2a), anti-CD7 antibodies 4H9 (IgG2a) and S91 (IgG2a) (obtained from Dr. E. Engelman, Stanford), the anti-MHC class II antibody L243 (IgG2a), and anti-B2 microglobulin antibody L368 (IgG1). The Thy-1.1 hybridoma was provided by Drs. Nowinsky and Bernstein (Fred Hutchinson Cancer Center, Seattle). The anti-idiotype antibodies, anti-TAB (IgG1) and anti-OCI LY8 (IgG2a), were produced by fusion of the spleen cells of BALB/c mice with P3X6 8653 myeloma cells. The mice were immunized with the secreted idiotype of the hybridoma between the TAB or OCI LY8 tumor cells and K6HB5 myeloma cells (13). All antibodies were purified by double ammonium sulfate precipitations and were judged to be greater than 80% pure on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The control nonbinding antibody used in the growth inhibition assays was the OCI LY8 anti-idiotype antibody for experiments using the cell lines CEM/VLB, TAB, and AKR/J SL2, and the TAB anti-idiotype antibody for experiments using OCI LY8.

Growth Inhibition. The targeting or control antibody was added at 0.1 μg/10^5 cells and after 20 min at 4°C, excess antibody was removed by washing with cold RPMI 1640 twice. Aliquots of 1×10^5 cells in 1 ml were added in duplicate to a 24-well plate. Increasing molar concentrations of PA liposomes or free drug in 10 μl were added to the appropriate wells. The drug or PA liposomes were incubated with the cells continuously for the entire assay period. The cells were incubated for 72 h at 37°C, 5% CO_2, and then counted on a Coulter Counter. All assays were performed in RPMI 1640 (Gibco) supplemented with 15% heat-inactivated fetal calf serum (Flow Laboratories). Results were expressed either as percentage of control cell number or as the IC_{50}.

Liposome Binding. Cells (1×10^6) were incubated 20 min at 4°C with the specific antibody (100 μl at 10 μg/ml per 10^6 cells). Excess antibody was removed by washing twice with Dulbecco's PBS with 1% bovine

serum albumin. The cells were resuspended in 1 ml PBS and PA liposomes were added and incubated at 4°C for 20 min. Excess PA liposomes were removed by washing twice and then 100 μl of the nonspecific fluorescein-conjugated antibody were added as a counterstain, then washed after 20 min at 4°C. Cells were resuspended in 1 ml PBS at 4°C and analyzed on the Becton Dickinson FACS 440.

Liposome Internalization. PA liposomes containing carboxyfluorescein were used to demonstrate liposome internalization. Cells (1×10^6/tube) were pretreated with appropriate antibodies and washed. The carboxyfluorescein PA liposomes were added at lipid concentrations equal to the predetermined IC_{50} and incubated at 4°C or 37°C for specified times, washed with cold medium, and then analyzed on the FACS.

Antibody Binding. The degree of antibody binding to the various cell lines was assessed by indirect immunofluorescence by using an affinity purified goat anti-mouse fluorescein-conjugated monoclonal antibody. Samples were analyzed on the FACS 440.

Protein A Binding. The degree of protein A binding was assessed by an ELISA in which 96-well microtiter plates (Immulon, Dynatech) were coated with protein A at a concentration of 5 μg/ml for 18 h. The plates were blocked with PBS containing 5% Carnation evaporated milk and then washed. Serial dilutions of the respective antibodies (pH 7.6) were added (1 μg/ml) and washed after a 1-h incubation. Goat anti-mouse immunoglobulin horseradish peroxidase was then added for 1 hour and washed. 2,2'-Azidobis(3-ethylbenzthiazolinesulfonic acid)-citric acid-H_2O_2 substrate solution was added, and color was quantitated on a Dynatech micro-ELISA reader at a wavelength of 405 nm with a reference wavelength of 630 nm.

RESULTS

Growth Inhibition. We assessed the ability of various cell-specific monoclonal antibodies linked to PA liposomes containing a drug to inhibit the growth of several malignant cell lines. Two different compounds were assessed. Initially, methotrexate-γ-aspartate was chosen for encapsulation. This pteridine antifolate is not as efficiently transported into cells as methotrexate, while it is an equally good inhibitor of dihydrofolate reductase (14). The effect from possible uptake of leaked drug is therefore insignificant (9, 15). Subsequently, methotrexate was tested as well.

Growth inhibition of B- and T-cell lines by PA liposomes with encapsulated methotrexate-γ-aspartate bound to a variety of antibodies is shown in Table 1. The IC_{50} for free nonencapsulated methotrexate-γ-aspartate was similar in the different human lines (8×10^{-7} to 1×10^{-6} M), while the murine line was slightly more sensitive (IC_{50} of 5×10^{-7}). The drug encapsulated in nontargeted PA liposomes was more effective in growth inhibition than free drug on all cell lines, with the greatest reduction of IC_{50} (7.5-fold) seen in the murine line. With the targeted PA liposomes, in the murine example as shown previously (9), there was a marked further reduction in the IC_{50} of 12.5-fold compared to nonspecific antibody-targeted PA liposomes. In the human lines, only a modest targeting effect was seen using the anti-MHC class II monoclonal antibody, L243 in one B-cell lymphoma line with an approximate 2-fold reduction in the IC_{50} compared to PA liposomes targeted with a nonspecific antibody. This reduction in IC_{50} was seen reproducibly in three separate experiments. No reduction in IC_{50} was seen when targeting was performed with an anti-idiotype antibody against this B-cell line or with antibodies against CD5 or CD7 in the T-cell line.

Because of the minimal growth inhibition seen in the human cell lines with most of the monoclonal antibodies tested, methotrexate, a chemotherapeutic agent more potent in free form than the γ-substituted derivative, was encapsulated into the PA liposomes. Table 1 also shows the IC_{50} for human cell lines when methotrexate instead of methotrexate-γ-aspartate was incorporated in the PA liposomes. The cell lines were clearly more sensitive to free methotrexate than to methotrexate-γ-aspartate. A targeting effect was again seen with the anti-MHC class II antibody. In this case there was a 3-fold reduction in IC_{50} for OCI LY8 and a 2-fold reduction for TAB. There was an increase in IC_{50} for encapsulated methotrexate as compared to free methotrexate which was not seen for encapsulated methotrexate-γ-aspartate as compared to free methotrexate-γ-aspartate. In contrast to methotrexate-γ-aspartate, free methotrexate is more effective than drug encapsulated into liposomes because of its higher rate of influx. However, the IC_{50} of encapsulated methotrexate is similar to that of encapsulated methotrexate-γ-aspartate, since cell entry of encapsulated drug depends on the uptake of the liposomes. The antibodies used for targeting did not alter the cytotoxicity induced by either free methotrexate or free methotrexate-γ-aspartate (data not shown).

Thus, specific growth inhibition in the human cell lines by PA liposomes containing methotrexate or methotrexate-γ-aspartate was seen only when an anti-MHC class II antibody was used for targeting the liposomes. Fig. 1 shows the PA liposome dose-response effect when the anti-MHC class II antibody was used for targeting. An antibody dose-response effect was also seen with this targeting antibody (data not shown). Note that the IC_{50} for free methotrexate was lower than the IC_{50} for anti-MHC class II antibody-targeted PA liposomes containing methotrexate. In this case enhancement of targeting refers to a reduction in IC_{50} for specifically targeted as compared to non-

Table 1 *Growth inhibition of leukemia cell lines by protein A liposomes*

Cells (1×10^5) were washed, reacted with designated antibodies (100 μl of 1 μg/ml), washed again, and exposed to varying molar concentrations of liposomes or free drug. After 72 h at 37°C, the cell numbers were counted on a Coulter Counter. Exposures to drug and PA liposomes were continual. No enhanced targeting effect was seen at shorter drug or liposome exposure times (data not shown).

Cell line	Antibody	IC_{50} (M)[a] Free drug	IC_{50} (M)[a] PA liposome
Methotrexate-γ-aspartate			
CEM/VLB (human T)		8×10^{-7}	
	Control[b]		2.5×10^{-7}
	Anti-CD7 (4H9)		2.5×10^{-7}
	Anti-CD7 (S91)		2.5×10^{-7}
	Anti-CD5 (Leu-1)		2.0×10^{-7}
TAB (human B)		1×10^{-6}	
	Control		8×10^{-7}
	Anti-idiotype		9×10^{-7}
	Anti-MHC class II (L243)		5×10^{-7}
AKR/J SL2 (murine T)		5×10^{-7}	
	Control		7.5×10^{-6}
	Thy-1.1		5×10^{-9}
Methotrexate			
CEM/VLB (human T)		8×10^{-9}	
	Control		5×10^{-7}
	Anti-CD5 (Leu-1)		5×10^{-7}
	Anti-B2 microglobulin		4×10^{-7}
TAB (human B)		2×10^{-8}	
	Control		6×10^{-7}
	Anti-idiotype		6×10^{-7}
	Anti-MHC class II (L243)		3×10^{-7}
OCI LY8 (human B)		4×10^{-8}	
	Control		9×10^{-7}
	Anti-idiotype		9×10^{-7}
	Anti-MHC class II (L243)		3×10^{-7}

[a] Mean standard deviation between duplicates in all experiments was less than 5%.

[b] Murine class matched nonbinding antibody.

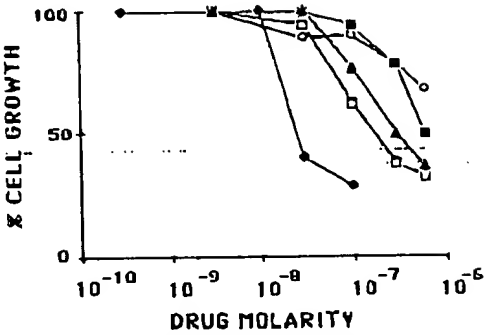


Fig. 1. Specific enhanced growth inhibition effect by class II antibody-targeted PA liposomes. Growth inhibition was assessed for the OCI LY8 cell line (see "Materials and Methods") and expressed as percentage of control cell growth. Specific enhanced growth inhibition was seen with both methotrexate and methotrexate-γ-aspartate containing PA liposomes (although results shown are with methotrexate encapsulated PA liposomes). Shown is a representative experiment with free methotrexate (◆), nonspecific antibody-PA liposomes (◇), anti-OCI LY 8 idiotype-PA liposomes (■), anti-MHC class II-PA liposomes (□), anti-idiotype and anti-MHC class II-PA liposomes (▲).

specifically targeted drug containing PA liposomes. For the other antibodies used on the human cell lines, the curves in Fig. 2 were representative of the absence of targeting effect seen over a 1000-fold increment in the concentration of the targeting antibody. Because of this result, several variables were examined to explain why only the anti-MHC class II antibody was effective in producing enhanced growth inhibition.

Comparison of Antibody Binding. A comparison of the fluorescent intensity of monoclonal antibody binding to the various cell lines was made in order to correlate antibody-directed PA liposome-mediated growth inhibition and antibody-cell binding. Table 2 shows the mean fluorescent intensity (using the same fluorescein-labeled second step antibody) for the various antibodies used for targeting on the different cell lines. The anti-MHC class II antibody bound well to both B-cell lines, although there were other antibodies that exhibited higher degrees of binding to these cell lines. No correlation between specific PA liposome growth inhibition effect and mean fluorescence was seen.

Comparison of Protein A Binding. To detect any correlation between specific monoclonal antibody directed PA liposome mediated growth inhibition and the ability of the monoclonal antibody to bind protein A, an ELISA binding assay was performed (Table 2). The antibody Thy-1.1 used on the AKR/J SL2 cell line eliciting a marked enhanced specific growth-inhibiting effect binds to protein A most avidly. There were several other antibodies which bound protein A well, yet only the anti-MHC class II antibody produced enhanced growth inhibition compared to nonspecifically targeted PA liposomes. Antibodies which did not bind to protein A, such as anti-B2 microglobulin antibody, regardless of brightness to which they bound to the target were not able to elicit specific enhanced PA liposome growth inhibition.

Demonstration of PA Liposome Binding. To demonstrate that the PA liposomes were binding to the cell via the targeting antibodies, an indirect assay was used, based on the binding of a fluoresceinated nonspecific antibody to "unoccupied" protein A sites on PA liposomes bound to the cell surface, Fig. 3 shows a shift in the mean fluorescence in both a murine and human cell line from the addition of a nonspecific fluorescein-conjugated IgG2a antibody to the cell-specific antibody-PA liposome complex. This shift did not occur when this antibody was added to either a cell-antibody (no PA liposome) complex or to a cell-PA liposome (no specific antibody) complex. A shift in mean fluorescence indicating PA liposome binding to the cell via the

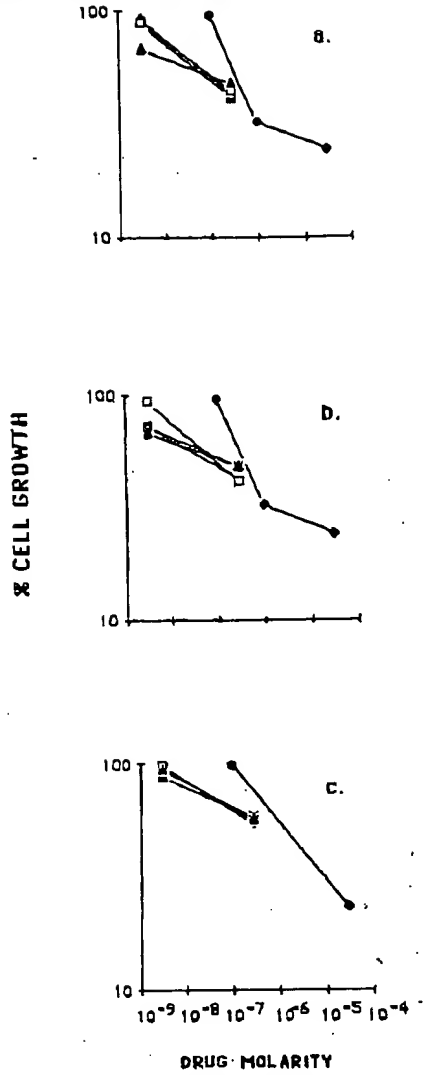


Fig. 2. Effect of targeting antibody concentration on growth inhibition. Cells (1×10^5) were washed and incubated with the varying antibody amounts at 4°C for 20 min, washed, and then exposed to varying molar concentrations of PA liposomes or free drug (methotrexate-γ-aspartate). After a 72-h incubation at 37°C, samples were counted on a Coulter Counter. Exposure to drug and PA liposomes was continual. Data are shown for the CEM cell line but are similar to data from other lines (see in Table 1) with antibodies that were unable to produce specific enhanced growth inhibition. In all curves, free methotrexate-γ-aspartate (◆), nonspecific antibody, 1 μg, PA liposome (▲). In *a*, *b*, and *c*, PA liposomes are targeted with S91, 4H9, and Leu-1, respectively, at 1 μg (◇), 0.1 μg (■), and 0.01 μg (□) (per 10^5 cells).

specific antibody occurred on the TAB cell line for PA liposomes targeted by both the anti-idiotype and anti-MHC class II antibodies. However, from the growth inhibition experiments discussed above, only the anti-MHC class II antibody resulted in specific growth inhibition. Thus, cell immunoglobulin-PA liposome binding can occur without enhancing growth inhibition.

The histograms (shown on a logarithmic scale) in Fig. 3 indicate that the degree of cell binding for PA liposomes targeted by the anti-idiotype antibody and the anti-MHC class II antibody was similar in magnitude to the amount of binding seen with the anti-Thy-1.1 antibody on the murine cell line. Thus, degree of PA liposome binding to the cell does not account for the marked differences in ability of the anti-Thy-1.1 antibody as compared to the other two antibodies to produce PA liposome mediated growth inhibition.

Demonstration of PA Liposome Internalization. In order to explain the discrepancy between binding and growth inhibition,

Table 2 *Comparison of degree of antibody binding to cells and antibody binding to protein A*

Cell line	Antibody	Antibody-cell binding (fluorescence)[a]	Antibody-protein A binding (A_{492})[b]
CEM/VLB	Anti-B2M	133	0.08
	Anti-CD5 (Leu-1)	70	0.363
	Anti-CD7 (4H9)	105	0.435
	Anti-CD7 (S91)	105	0.430
TAB	Anti-MHC class II	108	0.273
	Anti-TAB idiotype	64	0.15
OCI LY8	Anti-MHC class II	102	0.273
	Anti-OCI LY8 idiotype	145	0.246
AKR/J SL2	Anti-Thy-1.1		0.633

[a] Mean fluorescent intensity was assessed by FACS after indirect staining with a goat anti-mouse fluorescein conjugate. Binding is shown as mean fluorescent units minus control nonbinding antibody.

[b] Protein A binding was assessed by an ELISA assay, as per "Materials and Methods." Background absorbance, as detected on the detector antibody (low protein A binder) was subtracted from sample absorbance. Absorbance values shown represent a 1:16 dilution for all antibodies.

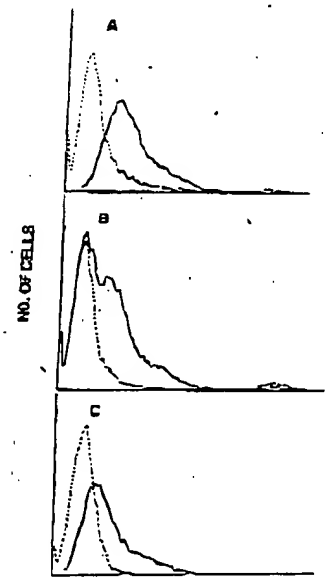


Fig. 3. Demonstration of liposome binding. A nonspecific fluoresceinated anti-idiotype antibody (IgG2a) was utilized to indicate PA liposome binding to cell in a successive layering assay. Specific or control antibody (1 μg/10^6 cells) was added to the cells at 4°C. PA liposomes were then added. After washing, indicator fluoresceinated nonspecific antibody was added and excess was washed off. ——, log scale fluorescence seen when all components of the layered complex were added; ——, decreased fluorescence seen when one of the components (specific targeting antibody or PA liposome) was excluded. PA liposomes targeted with (*A*) the Thy-1.1 antibody on the AKR/J SL2 cell line; (*B*) the anti-TAB idiotype antibody on the TAB cell line; (*C*) the anti-MHC class II antibody on the TAB cell line.

we determined whether the PA liposomes associated with cells were internalized. To do this we made use of the self-quenching effect of carboxyfluorescein when it is encapsulated at high concentrations within the internal aqueous spaces of liposomes (16, 17). The release of carboxyfluorescein into the cell, after successful uptake, results in measurable increased fluorescence, due to dilution and dequenching. The background immunofluorescence for cells binding carboxyfluorescein PA liposomes targeted with the antibodies at 4°C is shown in Fig. 4. Since the process of internalization is inhibited at 4°C, this fluorescence did not change significantly when assessed at 90 or 150 min (data not shown). At 37°C, there was an increase in mean fluorescence for the cells targeted with the anti-MHC class II

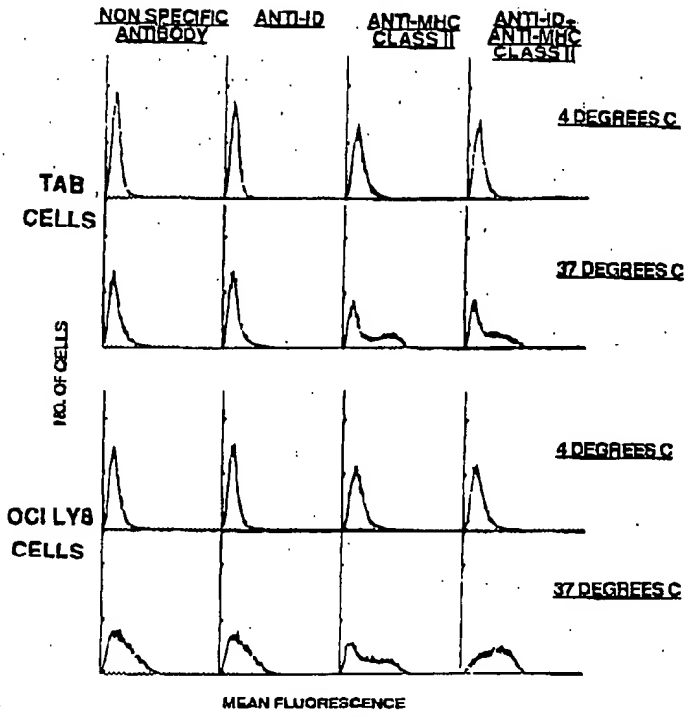


Fig. 4. Demonstration of liposome uptake. PA liposomes containing carboxyfluorescein were utilized to assess liposome internalization of two B-cell lines. The self-quenching effect of carboxyfluorescein at high concentration in the PA liposome is overcome when carboxyfluorescein is released into the cell. Cells (1×10^6) were reacted with antibody (100 μl of 10 μg/ml) for 20 min at 4°C, washed, and then the PA liposomes were incubated for 20 min at 4°C and washed. One set of samples was kept at 4°C (to minimize receptor-mediated endocytosis) and one set was incubated at 37°C, for 30 min. Samples were analyzed on the FACS 440. *ANTI-ID*, anti-idiotype.

antibody for both the OCI LY8 and the TAB cell lines. This indicates internalization of the liposome with subsequent release of carboxyfluorescein into the cytoplasm. This increase in fluorescence was not seen when targeting was performed with a nonspecific antibody or with anti-idiotype antibodies against either cell line. Thus, a correlation was seen between monoclonal antibody-directed PA liposome internalization and the ability of the antibody to produce specific PA liposome-mediated growth inhibition. It is noted that the greatest PA liposome internalization was seen in the OCI LY8 line when both the anti-MHC class II and anti-idiotype antibodies were used together for targeting. The further increase seen in liposome internalization for the two antibodies together did not result in further enhancement of growth inhibition compared to anti-MHC class II targeting alone (Fig. 1).

DISCUSSION

The data in this paper show that drug-containing small unilamellar liposomes which are conjugated to protein A can be targeted with a monoclonal antibody to result in specific growth inhibition of human B-cell lines (Table 1). Although examples are present in the literature that show enhanced cytotoxicity with targeted PA liposomes in murine cell lines (16, 18), to date specific liposome cytotoxicity in human lymphoma cell lines has been unsuccessful (8).

We have demonstrated that the enhanced growth inhibition seen by targeting with the anti-MHC class II monoclonal antibody correlates with an increase in PA liposome internalization (Fig. 4). This enhanced growth inhibition and PA liposome internalization does not occur with antibodies directed against

other cellular surface antigens such as the immunoglobulin idiotype. The degree of growth inhibition and PA liposome internalization seen with anti-idiotype antibodies and antibodies to T-cell differentiation antigens was equivalent to that seen with targeting by control nonbinding antibodies. This growth inhibition and internalization was probably related to nonspecific endocytosis of the PA liposomes on their own and not through an antibody-mediated pathway. This has been previously demonstrated for AKR/J SL2 cells with both direct antibody-liposome conjugates and PA liposomes (8, 9). Similar growth inhibition was seen when cells were incubated with PA liposomes without a targeting antibody (data not shown).

When the anti-MHC class II antibody was used for targeting, a dose-response effect was seen for both the amount of PA liposomes and the amount of targeting antibody used. In contrast, with all other antibodies used for targeting, no dose response could be seen over a 1000-fold range in targeting antibody concentration. Increasing amounts of antibody were thus unable to increase the intrinsically minimal antibody-mediated PA liposome internalization and growth inhibition by these antibodies. A concentration effect for antibody was thus seen with the antibody that was internalized well and not with those that were not internalized well. This is similar to results reported in a previous publication that used a murine model (8).

Although the enhancement of growth inhibition seen with the specific as compared to the nonspecific monoclonal antibody-targeted PA liposomes (approximately 3-fold) was not as dramatic as that seen with some monoclonal antibody-toxin conjugates (19, 20), a reproducible and specific effect was seen.

We have investigated some of the factors regulating the ability of monoclonal antibody targeted PA liposomes to produce a cell specific growth inhibitory effect. In order to understand why the specific growth inhibitory effect was seen with certain monoclonal antibodies (anti-MHC class II) and not with others (anti-idiotypes and antibodies to T-cell differentiation antigens), we assessed the degree of cell binding and protein A binding of the targeting antibody. Although the binding of the anti-MHC class II monoclonal antibody was high on both cell lines where a specific growth-inhibitory effect was seen (Table 2), the binding of some of the other monoclonal antibodies was similarly high. Similarly, protein A binding could not distinguish between antibodies which were able to mediate targeting and those which were not (Table 2). Clearly, for an antibody to enhance PA liposome-mediated growth inhibition, both cell surface binding and protein A binding must be above a critical level. However, these two factors alone did not assure that enhanced growth inhibition would result.

Despite the ability of PA liposomes to bind to cells via an anti-idiotype targeting antibody (Fig. 3), enhanced growth inhibition did not occur as it did with the anti-MHC class II antibody. The histograms in Fig. 3 show that the degree of PA liposome binding to the TAB cell line was comparable for targeting with either the anti-idiotype or anti-MHC class II antibodies. Furthermore, the degree of PA liposome binding through either of these antibodies was approximately as great as with the antibody which produces the most marked example of specific enhanced growth inhibition (anti-Thy-1.1 with the AKR/J SL2 murine cell line). However, increased PA liposome internalization did not occur with the anti-idiotype antibody as it did with the class II antibody (Fig. 4). The defect was clearly in PA liposome internalization for the anti-idiotype antibody PA liposome complex.

Antibody isotype is not a probable explanation for the differences in antibody-mediated liposome internalization. Although the TAB anti-idiotype was an IgG1 and the anti-MHC class II was an IgG2a, a defect in internalization was also seen with the OCI LY8 cell line and its IgG2a anti-idiotype (Fig. 4).

No additional effect on growth inhibition was seen when targeting was performed with both an antibody directed against a class II MHC determinant (where internalization was demonstrated) and an anti-idiotype antibody (where internalization on its own did not occur). The internalization data (Fig. 4) suggested that for the OCI LY8 cell line, more PA liposome internalization occurred when both the anti-idiotype antibody and the anti-MHC class II antibody were used together for targeting. The explanation for this discrepancy is unclear; however, the increase in internalization seen with the combination of antibodies may not be enough to cause augmented growth inhibition.

It is significant that only the antibody to the class II MHC determinant resulted in enhanced PA liposome internalization and growth inhibition in both B-cell lines tested. Other investigators have shown that PA liposomes targeted to class II determinants on murine B-cell lines result in increased liposome uptake and cellular cytotoxicity (7, 21). This may suggest a special role for class II determinants on B-cells for MHC-associated antigen endocytosis and processing.

An analogy can be drawn between specific growth inhibition with antibody-directed PA liposomes and antibody-toxin conjugates. Specific tumor cell lysis has been described with several different antibody-toxin conjugates, with the most marked examples being with immunotoxins directed against the immunoglobulin idiotype or isotype of a murine B-cell tumor (22, 23) or against the Thy-1 antigen on T-cells and T-cell leukemias (24–26). As with liposomes, variability in target cell sensitivity to immunotoxins has been documented (24, 27). Factors responsible for this variability have been described and include density of cell surface antigen (28), the rate of endocytosis of the antigen-immunotoxin complex (29), and the isotype of the targeting antibody (29, 30). Although we have not found the degree of targeting antibody binding (as measured by mean fluorescent intensity) and antibody isotype to be significant factors, our results concur with the importance of antigen-antibody endocytosis as a major parameter influencing effective liposome growth inhibition.

The specific growth inhibition seen by using anti-idiotype antibodies to target PA liposomes was not as marked as was seen by several authors when anti-idiotypes were used to target immunotoxins (21, 23, 31). Some immunotoxins are exquisitely effective in inhibiting cellular protein synthesis and only one ricin A chain can completely inhibit protein synthesis and kill the cell (32). Although we have shown that anti-idiotype antibodies were not internalized as rapidly as the anti-MHC class II antibody, some internalization was seen (Fig. 4). Probably, the difference in growth inhibition seen with anti-idiotype-targeted PA liposomes and anti-idiotype-targeted immunotoxins is stoichiometric with sufficient internalization occurring to deliver lethal amounts of immunotoxin but insufficient amounts of chemotherapeutic drug to kill the cell.

We have demonstrated that PA liposomes can be targeted with an anti-MHC class II monoclonal antibody in human B-cells to result in specific PA liposome internalization and cellular growth inhibition. A correlation between antibody-directed PA liposome internalization and enhanced growth inhibition was obtained. Cell surface and protein A binding of the targeting antibody are necessary but not sufficient factors for determining specific growth inhibition. The ability of the

targeted antigen to perform receptor-mediated endocytosis seems to be important and may limit the use of PA liposomes for cytotoxic purposes to certain targeting antibodies. Further clarification of these factors are necessary in order to optimize the utility of liposomes for specific cellular cytotoxicity.

ACKNOWLEDGMENTS

We are grateful to Dr. L. Leserman for his helpful advice and assistance and to Emily Chatfield, Phyllis Bussey, and Carolyn Wells for preparation of the manuscript.

REFERENCES

1. Mayhew, E., and Papahadjopoulos, D. Therapeutic applications of liposomes. *In:* M. J. Ostro (ed.) Liposomes, pp. 289–341. New York: Marcel Dekker, Inc., 1983.
2. Gregoriadis, G. Use of monoclonal antibodies and liposomes to improve drug delivery. Drugs, *24:* 261–266, 1982.
3. Weinstein, J. N., and Leserman, L. D. Liposomes as drug carriers in cancer chemotherapy. Pharmacol. Ther., *24:* 207–233, 1984.
4. Hashimoto, Y., Sugawara, M., Masuko, T., and Hojo, H. Antitumor effect of actinomycin D entrapped in liposomes bearing subunits of tumor-specific monoclonal immunoglobulin M antibody. Cancer Res., *43:* 5328–5334, 1983.
5. Kronvall, G., Grey, H. M., and Williams, R. C. Protein A reactivity with mouse immunoglobulins. J. Immunol., *105:* 1116–1123, 1970.
6. Martin, F. J., and Papahadjopoulos, D. Irreversible coupling of immunoglobulin fragments to preformed vesicles. J. Biol. Chem. *257:* 286–288, 1982.
7. Machy, P., Barbet, J., and Leserman, L. Endocytosis of T and B lymphocyte surface molecules evaluated with antibody-bearing fluorescent liposomes containing methotrexate. Proc. Natl. Acad. Sci. USA *79:* 4148–4152, 1982.
8. Matthay, K. K., Heath, T. D., Badger, C. C., Bernstein, I. D., and Papahadjopoulos, D. Antibody-directed liposomes: comparison of various ligands for association, endocytosis, and drug delivery. Cancer Res., *46:* 4904–4910, 1986.
9. Matthay, K. K., Heath, T., and Papahadjopoulos, D. Specific enhancement of drug delivery to AKR lymphoma by antibody-targeted small unilamellar vesicles. Cancer Res., *44:* 1880–1886, 1984.
10. Bartlett, G. R. Phosphorous assay in column chromatography. J. Biol. Chem., *234:* 466–468, 1959.
11. Carrell, W. L., Link, M. S., Smith, D., Bologna-Vaughan, S., Carswell, C., and Levy, R. Anti-idiotype antibodies in childhood B cell leukemia. Clin. Res., *34:* 848A, 1986.
12. Tweedale, M., Lin, B., Jamal, N., Minden, M., and Messner, H. A. Growth of lymphoma colonies: a predictive parameter for clinical outcome. J. Cell. Biochem., *9A:* 117, 1985.
13. Brown, S., Dilley, J., and Levy, R. Immunoglobulin secretion by mouse × human hybridomas. J. Immunol. *125:* 1037–1043, 1980.
14. Piper, J., and Montgomery, J. Synthesis of alpha- and gamma-substituted amides, peptides and esters of methotrexate and their evaluation as inhibitors of folate metabolism. J. Med. Chem., *25:* 182–187, 1982.
15. Heath, T. D., Montgomery, J. A., Piper, J. R., and Papahadjopoulos, D. Antibody-targeted liposomes. Proc. Natl. Acad. Sci. USA, *80:* 1377–1381, 1983.
16. Leserman, L. D., Machy, P., and Barbet, J. Cell specific drug transfer for liposomes bearing monoclonal antibodies. Nature (Lond.), *293:* 226–228, 1981.
17. Straubinger, R. M., Hong, K. Friend, D. S., and Papahadjopoulos, D. Endocytosis of liposomes and intracellular fate of encapsulated molecules. Cell, *32:* 1069–1079, 1983.
18. Heath, T. D., Macher, B. A., and Papahadjopoulos, D. Covalent attachment of immunoglobulins to liposomes via glycosphingolipids. Biochim. Biophys. Acta, *640:* 66–81, 1981.
19. Vitetta, E. S., and Uhr, J. W. Immunotoxins. Annu. Rev. Immunol., *3:* 197–212, 1985.
20. Vitetta, E. S., Krohck, K. A., Miyama-Inaba, M., Cushley, W., and Uhr, J. W. Immunotoxins: a new approach to cancer therapy. Science (Wash. DC), *219:* 644–650, 1983.
21. Aragnol, D., Malissen, B., Schiff, C., Piron, M. A., and Leserman, L. Endocytosis of MHC molecules by B cell-B lymphoma and B cell-T lymphoma hybrids. J. Immunol., *31:* 3347–3353, 1986.
22. Krolick, K. A., Villemez, L. Isakson, P., Uhr, J., and Vitetta, E. Selective killing of normal or neoplastic B cells by antibody coupled to the A chain of ricin. Proc. Natl. Acad. Sci. USA, *77:* 5419–5423, 1980.
23. Krolick, K. A., Uhr, J., Slavin, S., and Vitetta, E. *In vivo* therapy of a murine B cell tumor (BCL_1) using antibody-ricin A chain immunotoxins. J. Exp. Med., *155:* 1797–1809, 1982.
24. Thorpe, P. E., and Ross, W. The preparative and cytotoxic properties of antibody-toxin conjugates. Immunol. Rev., *62:* 119–158, 1982.
25. Myers, C., Thorpe, P., Ross, W., Cumber, A., and Katx, F. An immunotoxin with therapeutic potential in T cell leukemia: WT1-ricin A. Blood, *63:* 1178–1185, 1984.
26. Seon, B. K. Specific killing of human T leukemic cells by immunotoxins prepared with ricin A chain and monoclonal anti-human T-cell leukemia antibodies. Cancer Res., *44:* 259–264, 1984.
27. Vitetta, E. S., and Uhr, J. Immunotoxins: redirecting nature's poisons. Cell, *41:* 653–654, 1985.
28. Casellas, P., Curriere, D., Gros, O., Laurent, J. C., Poncelet, P., and Jansen, F. Antibody-ricin A chain conjugates (immunotoxins) in specific cell killing. *In:* J. E. Alout, F. J. Fehrenback, J. H. Freer, and J. Jeljaszewicz (Eds.), Workshop conference on bacterial protein conjugates, Seillac, France, 1983, pp. 227–235. London: Academic Press, 1983.
29. Casellas, P., Bourrie, B., Gros, P., and Jansen, F. Kinetics of cytotoxicity induced by immunotoxins: enhancement by lysosomotrophic amines and carboxylic ionophores. J. Biol. Chem., *259:* 9359–9364, 1984.
30. Masuho, Y., Kishida, K., Saito, M., Umemoto, M., and Hara, T. Importance of the antigen-binding valency and the nature of the cross linked bond in ricin A-chain conjugates with antibody. J. Biochem., *91:* 1583–1591, 1982.
31. Gregg, E. O., Bridges, S. H., Youle, R. J., Longo, D. L., Houston, L. L., Glennie, M. J., Stevenson, F. K., and Green, I. Whole ricin and recombinant ricin A chain idiotype-specific immunotoxins for therapy of the guinea pig L_2C B cell leukemia. J. Immunol., *138:* 4502–4508, 1987.
32. Eiklid, K., Olsnes, S., and Pihl, A. Entry of lethal doses of abrin, ricin, and modeccin into the cytosol of HeLa cells. Exp. Cell. Res., *126:* 321–326, 1980.